A 54-kDa Fragment of the Poly(A)-specific Ribonuclease Is an Oligomeric, Processive, and Cap-interacting Poly(A)-specific 3' Exonuclease*

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We have previously identified a HeLa cell 3' exonuclease specific for degrading poly(A) tails of mRNAs. Here we report on the purification and identification of a calf thymus 54-kDa polypeptide associated with a similar 3' exonuclease activity. The 54-kDa polypeptide was shown to be a fragment of the poly(A)-specific ribonuclease 74-kDa polypeptide. The native molecular mass of the nuclease activity was estimated to be 180–220 kDa.

Protein/protein cross-linking revealed an oligomeric structure, most likely consisting of three subunits. The purified nuclease activity released 5'-AMP as the reaction product and degraded poly(A) in a highly processive fashion. The activity required monovalent cations and was dependent on divalent metal ions. The RNA substrate requirement was investigated, and it was found that the nuclease was highly poly(A)-specific and that only 3' end-located poly(A) was degraded by the activity. RNA substrates capped with m^7G(5')ppp(5')G were more efficiently degraded than noncapped RNA substrates. Addition of free m^7G(5')ppp(5')G cap analogue inhibited poly(A) degradation in vitro, suggesting a functional link between the RNA 5' end cap structure and poly(A) degradation at the 3' end of the RNA.

In recent years it has become clear that poly(A) removal is an important step during mRNA decay, and it has been found that mRNA degradation in many cases is initiated by degrading the mRNA poly(A) tail (reviewed in Ref. 1). In yeast two major deadenylation-dependent pathways of mRNA degradation have been defined, the deadenylation-dependent decapping pathway and the 3'-5' decay pathway (for reviews see Refs. 2 and 3). The former pathway is initiated by poly(A) tail removal followed by decapping and 5'-3' exonucleolytic degradation of the decapped mRNA by the Xrn1p 5' exonuclease. The latter is initiated by deadenylation followed by 3'-5' exonucleolytic degradation of the mRNA. The participating 3'-5' exoribonucleases have not yet been unambiguously identified. A multicomponent complex, termed the exosome (4), has been identified in yeast as well as in human cells, and it contains at least 10 different 3'-5' exoribonucleases (reviewed in Ref. 5). The exosome is involved in 3' end processing of several stable RNAs (4, 6) and 3'5' degradation of mRNA (7). Thus, these exoribonucleases could be responsible for the degradation of mRNA including the poly(A). However, it remains to be established whether any of these exonucleases preferentially or exclusively degrade poly(A) of mRNA.

In mammalian cells mRNA decay has been studied extensively, and it has been found that gene expression to a large extent is regulated by changing the stability of an mRNA. From these studies it has been shown that cis-acting elements located in the mRNA body are important elements involved in regulating mRNA decay. One of the first elements to be identified was an AU-rich element located in the 3'-untranslated region of the human lymphokine granulocyte macrophage colony-stimulating factor mRNA (8). Shaw and Kamen (8) showed that the AU-rich element destabilized an mRNA and that the element could confer the destabilization effect if it was transferred to another mRNA. Furthermore, it has been found, equivalent to the case in yeast, that mRNA decay in mammalian cells in many cases is initiated by poly(A) removal (9). However, most of these studies (1) have been performed in vivo, and therefore, it has been difficult to identify participating components and to address mechanistic details. In the last years several in vitro cell-free mRNA stability systems have been developed, and these systems will make it possible to elucidate mechanistic details and to identify factors regulating mRNA decay in mammalian cells (10–14). Using such a system Wilusz and colleagues (13) have been able to stimulate poly(A) removal and RNA degradation by the presence of an AU-rich sequence element (8) in the RNA body and to reproduce regulated aspects of mRNA decay. Similarly, Brewer (11) has shown that the 3'-untranslated region of c-Myc affects poly(A) removal and RNA degradation in a different in vitro system. The poly(A)-specific exoribonucleases responsible for degrading the mRNA poly(A) tails in these in vitro decay systems remains to be identified.

Poly(A) tail removal and poly(A) degrading nuclelease activities have been studied in several eukaryotic systems (for reviews see Refs. 1, 3, and 15). In mammalian cells several different poly(A) degrading activities have been characterized over the years. Earlier studies (reviewed in Ref. 16) were hampered by the lack of RNA substrates resembling polyadenylated mRNA, making it difficult to assay, purify, and characterize nucleases that specifically degraded only the poly(A) tail of an mRNA. By the use of an in vitro transcribed and polyadenylated mRNA mimic we were able to define a poly(A)-specific 3' exonuclease in HeLa cell free extracts (17) and propose a reac-
tion pathway for mRNA poly(A) tail removal (18). Important properties of the HeLa cell activity were its high selectivity for degrading only 3'-located poly(A) tails, its requirement for a 3'-located hydroxyl group, and release of 5'-AMP as the mono-
nucleotide product. Recently, a poly(A)-specific ribonuclease (PARN) associated with a 74-kDa polypeptide was purified and characterized in calf thymus extracts by Körner and Wahle (19). Human PARN has been molecularly cloned, and PARN activity has been recovered from recombinant 74-kDa polypeptide (20). PARN has also been found in Xenopus and shown to play a role in poly(A) tail removal during meiotic maturation of Xenopus oocytes (20, 21). A polyribosome-associated 3’ exoribonuclease, having a molecular mass of 33 kDa has been de-
scribed and purified by Caruccio and Ross (22). However, this 3’ exoribonuclease is not specific for poly(A), although poly(A) can be degraded. In addition to these metazoan poly(A) degrad-
ing nucleases Sachs and colleagues have described a poly(A)-
degradation process that has been described and purified from 70 °C. Starting with 2.8 kg of calf thymus the obtained extract (number 500-0001) and bovine 
globulin as reference.

PREPARATION OF CELL-FREE EXTRACTS—All steps were performed at 4 °C. Crude whole cell extract was prepared from bovine calf thymus, essentially according to Wahle (29). In short, frozen calf thymus (2–15 kg) obtained from a local slaughter house was thawed on ice, cut into pieces, homogenized in an approximately equal volume of buffer 1 (50 mM Tris-HCl, 10 mM K3PO4, 1 mM EDTA, 10% glycerol, 50 mM KCl, 0.1 mM dithiothreitol, 20% glycerol at pH 7.5) and then treated as for the previous precipitation step. The supernatant was added to 2 liters of buffer D containing 25 mM KCl at pH 7. The centrifugation was applied to the column at flow rate of 1 ml/min. The column was first washed with 5 bed volumes of buffer D containing 250 mM KCl at pH 7. The protein concentration was determined using the Bio-Rad protein assay kit (number 500-0001) and bovine γ globulin as reference.

EXPERIMENTAL PROCEDURES

Preparation of Cell-free Extracts—All steps were performed at 4 °C. A typical protocol starting with 400 ml of crude ammonium sulfate fraction was frozen in liquid nitrogen and stored at 70 °C.

5'-AMP-Sepharose and 7-Methyl-GTP-Sepharose Affinity Chromatography—All steps were performed at 4 °C. A standard protocol is given below. The 5'-AMP-Sepharose 4B matrix (Amersham Pharmacia Biotech, 17-0830-01) was prepared according to the manufacturer. A HR10/10 column with bed volume of 2 ml was equilibrated with buffer D containing 25 mM KCl at pH 7. 12 ml of the Blue-Sepharose CL-6B fraction was dialyzed for 4 h against 2 × 2 liters of buffer D containing 25 mM KCl at pH 7. The dialyzed fraction was applied to the column at flow rate of 1 ml/min. The column was first washed with 5 bed volumes of buffer D containing 25 mM KCl at pH 7 followed by a second wash with 5 bed volumes of buffer D containing 200 mM KCl at pH 6. Subsequently, the column was washed with a gradient (5 bed volumes) from 280 to 600 mM KCl at pH 6. Poly(A)-specific exonuclease activity eluted between 300 and 550 mM KCl. This procedure purifies the specific activity of the poly(A)-specific exonuclease approximately 14-fold.

6 The abbreviations used are: PARN, poly(A)-specific ribonuclease; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MALDI-TOF, matrix-assisted laser desorption ionization time of flight.
column volumes) with buffer D at pH 7 supplemented with 50 mM KCl. The bound protein fraction was eluted with a salt step (10 column volume of buffer D supplemented with 2 mM KCl). The nuclease activity was identified (eluted during the 2 mM KCl step) and pooled (−0.750 μg of protein as determined by SDS-PAGE analysis and silver staining, 5 mM KCl, 5% (v/v) poly(vinyl alcohol) (Sigma P-8136; molecular weight, 10,000), 100 mM KCl, 0.15 units of RNAguard, 5–20 fmol of RNA substrate, 10 mM HEPES-KOH, pH 7, 0.1 mM EDTA, 0.25 mM dithiothreitol, 10% glycerol, and the indicated amount of protein fraction (18). The concentration of the nuclease activity in the purest 5‘-AMP-deadenylation fraction was approximately 100 units/mg. The RNA substrate was radioactively labeled as indicated. Reaction volume was 15 or 25 μl, and incubations were performed at 30 °C. Reactions were terminated, and the reaction products were investigated either by purifying the RNA and subsequent electrophoresis in 10% polyacrylamide (19:1 acrylamide:bisacrylamide)/7% urea gels as described previously (17, 18) or by one-dimensional TLC analysis (see below).

**SDS-Polyacrylamide Gel Electrophoresis and Renaturation of Nuclease Activity—**SDS-polyacrylamide gel electrophoresis (acrylamide:bisacrylamide 30:08 gels (5 and 7.5% acrylamide in spacer and separation gels, respectively) were prepared according to Laemmli (31) using a Mini-Protean II gel apparatus (Bio-Rad, 125BR). The indicated amount of protein was subjected to in-gel digestion as described (35). In brief, after washing with ammonium bicarbonate and acetonitrile, the gel piece was completely dried, and a solution containing modified porcine trypsin, sequence grade (Promega Corp. Madison, WI) was allowed to soak into the gel piece. After overnight incubation at 30 °C, generated peptides were recovered by extraction. The peptide mixture was analyzed by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, using a Bruker Biflex III instrument (Bremen, Germany), equipped with delayed extraction and reflector. The sample was prepared by the dried droplet technique, using a-cyano-4-hydroxycinnamic acid as matrix. The instrument was externally calibrated using angiotensin II (MH = 1046.54) and adrenocorticotropic hormone fragment 18-39 (MH = 2465.20). The peptide mass fingerprinting analysis was done using ProFound (version 4.7.5).

**Analytical SMART Superdex 200 Gel Filtration**—The pol(yA)-Sepharose fraction (0.7 ml, 0.06 mg/ml protein) was first concentrated by centrifugation (10,000 × g) PC. 1.6/5 (Amersham Pharmacia Biotech and chromatography by the following procedure. The column was equilibrated by buffer D at pH 7 containing 50 mM KCl. The pol(yA)-Sepharose fraction was dialyzed against buffer D containing 50 mM KCl at pH 7 and applied to the MonoQ column at flow rate 50 μl/min. Bound material was eluted by increasing the KCl to 500 mM, and fraction size was 25 μl. Active fractions (total volume, 100 μl) were identified and pooled. Subsequently, 50 μl of the concentrated pol(yA)-sepharose fraction was fractionated by gel filtration using a SMART Superdex 200 PC 3.2/30 column equilibrated with buffer D at pH 7 containing 100 mM KCl. Flow rate was 40 μl/min. Active fractions were identified by in vitro deadenylation. Molecular size markers were fractionated by the same procedure on the Superdex 200 column. Molecular size markers were prepared according to Laemmli (31) using a Mini-Protean II gel apparatus (Bio-Rad, 125BR). The indicated amount of protein was applied to the gel piece was completely dried, and a solution containing modified porcine trypsin, sequence grade (Promega Corp. Madison, WI) was allowed to soak into the gel piece. After overnight incubation at 30 °C, generated peptides were recovered by extraction. The peptide mixture was analyzed by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, using a Bruker Biflex III instrument (Bremen, Germany), equipped with delayed extraction and reflector. The sample was prepared by the dried droplet technique, using a-cyano-4-hydroxycinnamic acid as matrix. The instrument was externally calibrated using angiotensin II (MH = 1046.54) and adrenocorticotropic hormone fragment 18-39 (MH = 2465.20). The peptide mass fingerprinting analysis was done using ProFound (version 4.7.5).

**Molecular Cloning and Expression**—The 74-kDa PARN polypeptide and a 54-kDa fragment of PARN were molecularly cloned by a standard reverse transcription-polymerase chain reaction procedure followed by subcloning into the pGEM T vector (Promega Inc.). Inserts were cloned into the pET-19 vector (Novagen Inc.) between the NdeI and BamHI sites. The following primer pairs were used 5‘-TCGCGATATG-AGATAATCGGAGCA-3‘ and 5‘-TCGACATTTTACATCTTCAGG-AACTCTA-3‘ for the 74-kDa PARN polypeptide and 5‘-TCGCAAT-GAGGATACGAGGACA-3‘ and CTGGGATCTCAGTTAACAGG-GACTGGA for the 54-kDa fragment. RNA template was obtained from HeLa cells. Recombinant and His-tagged polypeptides were expressed in the Escherichia coli strain BL21(DE3) and extracted according to the manufacturer of the pET vectors. Recombinant polypeptides were purified by metal affinity chromatography using the TALON matrix (CLONTECH Inc. number 8901) as outlined by the manufacturer of the matrix.

**Poly(protein) Cross-linking**—Bis(sulfosuccinimidyldimethyl) pimelimidate were purchased from Pierce, and cross-linking was performed according to the instructions of the manufacturer. Essentially, indicated amounts of purified recombinant polypeptides, in buffer D at pH 8.2, were mixed with 10 mM cross-linking reagents and incubated for 60 min at room temperature. Total volume was 100 μl. The reactions were terminated by the addition of 11 μl of 1X Tris-HCl, pH 7.9.

**Two-dimensional Thin Layer Chromatography**—Chromatography on polyethyleneimine-cellulose F plates (Merck, number 5579) was performed according to Konarska et al. (36). Liberated products from deadenylation reactions were analyzed by two-dimensional TLC in standard chambers using: 0.75 μl KH₂PO₄, pH 3.5 (H₂PO₄), as first dimension solvent and isobutyric acid/concentrated NH₄OH/H₂O 577:38:385 (v/v) as second dimension solvent. 5’-AMP (Sigma, A-1732), 2’-AMP (Sigma, A-9396), and 3’-AMP (Sigma, A-0396) were included as reference compounds. These were detected by a light-emitting diode light (UVG-54, Ultra-Violet Products Inc.). Radioactive molecules were detected by autoradiography or by 400 S PhosphorImager (Molecular Dynamics) analysis of the resulting polyethyleneimine-cellulose F plate.

**One-dimensional TLC and Quantification of Nuclease Activity—**Deadenylate activity was quantified as follows: L3(A30) RNA substrate labeled with [³²P]ATP during in vitro transcription was incubated in conditions for in vitro deadenylation as described above with the addition of 0.1 mg/ml BSA. Reactions were analyzed by one-dimensional TLC using 0.75 μl KH₂PO₄, pH 3.5 (H₂PO₄), as the solvent. The resulting polyethyleneimine-cellulose F plate was dried and scanned by a 400 S PhosphorImager (Molecular Dynamics). The fraction of released [³²P]AMP was determined. Knowing the specific activity of [³²P]AMP in the RNA substrate, the amount of released AMP was calculated. One unit of deadenylate activity was defined as the release of 1 nmol of AMP/min.

**RESULTS**

**Partial Purification**—To purify a pol(yA)-specific 3′ exonuclease activity, we used a dual assay strategy that monitored both the disappearance of the RNA substrate and the appearance of the two reaction products: deadenylated RNA and released AMP. Disappearance of the RNA substrate and accumulation of the deadenylated product were investigated by in vitro deadenylation using body-injected L3(A30) RNA substrates followed by analytical polyacrylamide gel electrophoresis of the reacted RNA. Release of AMP was investigated by in vitro deadenylation of L3(A30) RNA substrate radioactively labeled in its poly(A) tail followed by detection of released mononucleotides by TLC. An important advantage of this dual assay strategy is that nucleases that degrade both the pol(yA)-
tail and the RNA body of the substrate can be excluded.

The poly(A)-specific 3' exonuclease activity was first partially purified from calf thymus whole cell extract using ammonium sulfate precipitation followed by four chromatographic steps (see “Experimental Procedures,” Table I, and Fig. 1). Using this purification protocol, a poly(A) removing activity fulfilling the requirements of the dual assay strategy was purified approximately 14,000-fold (Table I).

The partially purified Blue-Sepharose fraction was further purified by poly(A)-Sepharose affinity chromatography. This affinity step improved the purity of the poly(A)-specific exonuclease approximately 14-fold. The protein profile of the affinity step improved the purity of the poly(A)-specific exonuclease approximately 14,000-fold (Table I). The partially purified Blue-Sepharose fraction was further purified by poly(A)-Sepharose affinity chromatography. This affinity step improved the purity of the poly(A)-specific exonuclease approximately 14-fold. The protein profile of the obtained poly(A)-Sepharose fraction, as detected by SDS-PAGE, revealed several polypeptides (Fig. 2A). To determine whether any of those was associated with the activity, we eluted them from the SDS-PAGE gel matrix and subsequently denatured and renatured the eluted polypeptides. Exonuclease activity specific for degrading poly(A) and releasing AMP was recovered from polypeptides in the 50–60-kDa range (Fig. 2B and data not shown), suggesting that a polypeptide with this size was responsible for the poly(A)-specific 3' exonuclease activity.

A 54-kDa Polypeptide Is Responsible for the Activity—The Blue-Sepharose fraction was further purified by two consecutive steps of affinity chromatography, 5'-AMP-Sepharose followed by 7-methyl-GTP-Sepharose. SDS-PAGE of the final fraction revealed a prominent 54-kDa polypeptide (Fig. 3). The mass spectrum revealed ten peptides that were used to identify the putative 54-kDa polypeptide. Six of the ten peptides were located in human PARN, amino acids 87–99, 236–243, 260–272, 350–359, 414–421, and 444–454 (amino acids numbered according to Ref. 20).

The existence of the remaining four peptides is most likely explained by differences between the human and bovine PARN amino acid sequences. We conclude that the poly(A)-specific 3' exonuclease activity that we purified was associated with a 54-kDa fragment of the 74-kDa PARN polypeptide.

**Oligomeric Structure**—The 7-methyl-GTP-Sepharose fraction was analyzed by gel filtration using a SMART Superdex 200 column as the matrix. Active fractions were identified (Fig. 4A). The native molecular mass of the 54-kDa active fragment of PARN was estimated to 180–220 kDa. The poly(A)-Sepharose fraction was also analyzed by SMART Superdex 200 chromatography. The native molecular size was also in this case estimated to be 180–220 kDa. Both preparations of the 54-kDa active fragment of PARN eluted consistently between the 158-kDa aldolase and the 232-kDa catalase molecular size markers. The big discrepancy between the molecular masses estimated by gel filtration and SDS-PAGE suggests that the nuclease associated with the 54-kDa active fragment of PARN is oligomeric. To further investigate the oligomeric structure of the nuclease, we molecularly cloned and expressed recombinant human 74-kDa PARN polypeptide and human 54-kDa PARN fragment. The 74-kDa polypeptide was found to be expressed in the soluble fraction of the bacterial host, whereas the 54-kDa fragment was present in inclusion bodies. The human 74-kDa PARN was affinity purified, and poly(A)-specific nuclease activity was recovered (data not shown). The purified recombinant human 74-kDa PARN nuclease was subjected to protein/protein cross-linking using the homobifunctional cross-linkers bis(sulfosuccinimidyl) suberate and dimethyl pimelidimate. The cross-linked polypeptides were fractionated by SDS-PAGE and subsequently revealed by either silver staining of the gel (data not shown) or by Western

* TABLE I

| Purification of poly(A)-specific 3' exonuclease | Purification of poly(A)-specific 3' exonuclease |
|-----------------------------------------------|-----------------------------------------------|
| Step                                           | Protein Activity Specific activity Yield Purification |
| Extract                                       | 103,000 | 9,303 | 0.090 | 100 | 100 |
| Ammonium sulphate                             | 13,720 | 6,203 | 0.452 | 67 | 5 |
| DEAE-Sepharose                                 | 1,676 | 12,020 | 7.17 | 129 | 79 |
| Heparin-Sepharose                              | 441 | 2,283 | 5.18 | 25 | 57 |
| Mono Q                                        | 81.8 | 2,526 | 30.9 | 27 | 340 |
| Blue-Sepharose                                | 7.88 | 10,177 | 1,291 | 109 | 1,420 |
| 5' AMP/7-Me-GTP*                               | 0.003 | 633 | 211,000 | 7 | 2,321,000 |

* One quarter of the Blue-Sepharose fraction (1.97 mg protein) was taken through this step of purification as described under “Experimental Procedures.”
from the gel slices and subsequently denatured and renatured (34). Were cut out from a similar preparative gel in which 0.7 ml of the renatured proteins isolated from gel slices in vitro deadenylation for 90 min. In each reaction 12 μl of renatured proteins from a total volume of 1 ml of renatured protein was used. Reacted RNA was recovered and fractionated by electrophoresis using a 10% polyacrylamide:bisacrylamide 19:1–7M urea gel. The resulting flourogram is shown. Arrows to the right indicate the location of RNA substrate (S) and product (P).

Fig. 3. SDS-PAGE of proteins used for mass spectrometry. Lane 7MeG, proteins (0.75 μg in 1.65 ml) in fraction 5’-AMP/7-methyl-AMP/7-methyl-GTP was precipitated and fractionated by SDS-PAGE. The resulting gel was stained by silver. A gel slice containing the 54-kDa protein (marked with an arrow) was cut out from the gel and subsequently digested with trypsin. Tryptic peptides were analyzed by MALDI-TOF mass spectrometry. Molecular mass markers were separated in lane M. Numbers to the right indicate molecular masses of marker proteins in kDa.

The nuclease is oligomeric. A, the 5’-AMP/7-methyl-GTP fraction was fractionated by SMART Superdex 200 chromatography as described under “Experimental Procedures.” Obtained fractions (14–24) were incubated together with uniformly labeled RNA substrate L3(A30) under conditions for in vitro deadenylation for 120 min. In lane L the RNA substrate was incubated with the 5’-AMP/7-methyl-GTP fraction. Reacted RNA was recovered and fractionated by electrophoresis using a 10% polyacrylamide:bisacrylamide 19:1–7M urea gel. The resulting flourogram is shown. Arrows to the right denote the locations of RNA substrate (S) and product (P). Arrows at the top indicate the elution profile during SMART Superdex 200 chromatography of the molecular mass markers. Molecular mass is given in kDa. B, recombinant human 74-kDa PARN polypeptide (4 or 10 μg, as indicated, in a 100-μl cross-linking reaction, respectively) was treated with indicated homobifunctional cross-linkers bis(sulfosuccinimidyl) suberate and di-methyl pimelimidate as outlined under “Experimental Procedures” and fractionated by SDS-PAGE. The gel was subsequently analyzed by Western blot analysis using a His-tag-specific antiserum as the probe. The resulting flourogram is shown. Arrows to the left indicate the positions of forms I, II, and III. Numbers to the right indicate the position of molecular size markers in kDa. S/S denotes the boarder between the stacking and separation gels.

Conditions for in Vitro Deadenylation—The conditions for poly(A) tail removing activity were investigated by performing standard in vitro deadenylation reactions using the poly(A)-Sepharose fraction as the enzyme source and poly(A)-tailed labeled L3(A30) RNA as the substrate. Activity was monitored by following the release of mononucleotides by the one-dimensional TLC assay. The requirements for monovalent (K+ and Na+) and divalent (Mg2+, Mn2+, Zn2+, and Ca2+) cations were investigated. We found that monovalent cations were required and that the optimal concentration was around 100 mM. Poly(A) removing activity was higher in the presence of K+ than in the presence of Na+. The nuclease activity was dependent on divalent cations, and Mg2+ was found to be the preferred divalent ion with an optimal concentration around 1 mM. The nuclease was active in the presence of Mn2+. However, the activity decreased to 17% of the nuclease activity detected in the presence of Mg2+. No activity was detected in the presence of Zn2+ and Ca2+.
accumulation of the RNA body (Fig. 5A), whereas RNA substrates ML40(G14) and ML40(C32) were almost unaffected by the 3' exonuclease activity in both fractions (Fig. 5A and data not shown). Secondly, the RNA substrate specificity was investigated by incubating RNA substrates having internal poly(A) stretches followed by plasmid encoded RNA sequences of increasing length with either the poly(A)-Sepharose fraction or the final 7-methyl-GTP fraction under conditions for in vitro deadenylation. It was found that RNA substrates L3(A30), L3(A30)X15, L3(A30)X10, and L3(A30)X164 were almost unaffected by the 3' exonuclease activity in both preparations in contrast to the RNA substrate L3(A30), which was efficiently deadenylation (Fig. 5B). Finally, RNA substrates L3(A30), ML54(U100), ML40(C14), and ML43(G14) radioactively labeled in their homopolymeric tails were incubated in conditions for in vitro deadenylation using the poly(A)-Sepharose fraction. The release of radioactive mononucleotides was monitored by one-dimensional TLC assay. From this analysis we determined the apparent Km and Vmax values for each RNA substrate using Lineweaver-Burk formalism. Table II summarizes the obtained Km values and the calculated relative Vmax/Km values. Interestingly, the Km values for the different substrates were all in the 10 nM range, suggesting that substrate specificity is related to the Vmax parameter. Based on these three sets of experiments, we conclude that the nuclease activity associated with the 54-kDa active fragment of PARN is highly specific for degrading poly(A) and that it preferentially degrades 3’-end-located poly(A) tails.

5’-AMP Is the Released Mononucleotide—The nature of the released mononucleotide reaction product was investigated by two-dimensional TLC. The poly(A)-Sepharose fraction or the final 7-methyl-GTP fraction was incubated, under condition for in vitro deadenylation, together with RNA substrate L3(A30) radioactively labeled in its homopolymeric adenosine tail. The reaction products were analyzed by two-dimensional TLC using nonlabeled 2'-AMP, 3'-AMP, and 5'-AMP as markers. The result (Fig. 6 and data not shown) showed that the released mononucleotide comigrated with 5’-AMP. We conclude that 5’-AMP is a reaction product and that an exonuclease activity is responsible for the degradation.

The Nuclease Activity Is Highly Processive—To investigate whether the nuclease activity degraded RNA substrates in a processive or distributive fashion, we first titrated the exonuclease, using the final 7-methyl-GTP-Sepharose fraction as the source of enzyme and the RNA L3(A30) labeled in its body as the substrate. Incubation time was 10 min. Fig. 7A (lanes 1–6) shows that nonreacted RNA substrate and fully deadenylated RNA product were present when a low amount of exonuclease was added. Next, six identical reactions using 2 μl of the 7-methyl-GTP fraction as the exonuclease source were again incubated for 10 min, but in this case an increasing amount of poly(A) was added to each reaction. Fig. 7A (lanes 7–12) shows that addition of poly(A) inhibited the reaction and that both nondeadenylated and completely deadenylated RNA substrates were present when the concentration of poly(A) was 40 pg/μl or 0.4 ng/μl (lanes 10 and 11). The same results were obtained using the partially purified poly(A)-Sepharose frac-
FIG. 6. The liberated reaction product during deadenylation is 5′-AMP. The 5′-AMP/7-methyl-GTP fraction was incubated under conditions for in vitro deadenylation together with RNA substrate L3(A30), labeled by the inclusion of [32-P]GMP during in vitro transcription. A fraction of the reaction was subjected to two-dimensional TLC. The resulting autoradiogram of the dried polyethyleneimine-cellulose plate is shown. The location of 2′-AMP, 3′-AMP, and 5′-AMP markers are indicated.

Fig. 7. The nuclease activity is highly processive. The 5′-AMP/7-methyl-GTP fraction was incubated with RNA substrate L3(A30), labeled by the inclusion of [32-P]GTP during in vitro transcription in 25-μl reactions under conditions for in vitro deadenylation. Reacted RNA was purified and fractionated by electrophoresis in a gel containing 10% polyacrylamide/bisacrylamide 19:1–7 μurea. The resulting fluorograms are shown. Arrows to the right denote the locations of RNA substrate (S) and product (P). A, the RNA substrate concentration was 0.7 nM, and reactions were incubated for 10 min. Reactions fractionated in lanes 1–6 were performed in the presence of an increasing amount of the 5′-AMP/7-methyl-GTP fraction. The added amount was 0 μl (lane 1), 0.1 μl (lane 2), 0.3 μl (lane 3), 1 μl (lane 4), 2 μl (lane 5), and 4 μl (lane 6) reactions fractionated in lanes 7–12 were performed in the presence of 2 μl of the 5′-AMP/7-methyl-GTP fraction in an increasing amount of added poly(A). The final concentrations of added poly(A) were 0 pg/μl (lane 7), 0.4 pg/μl (lane 8), 4 pg/μl (lane 9), 40 pg/μl (lane 10), 0.4 ng/μl (lane 11), and 4 ng/μl (lane 12). B, the RNA substrate concentration was 7 nM (lanes 1–10) and 35 nM (lanes 11–20) in the presence of 2 μl of the 5′-AMP/7-methyl-GTP fraction. Reactions were terminated after 0 (lanes 1 and 11), 2 (lanes 2 and 12), 5 (lanes 3 and 13), 10 (lanes 4 and 14), 15 (lanes 5 and 15), 20 (lanes 6 and 16), 25 (lanes 7 and 17), 30 (lanes 8 and 18), 35 (lanes 9 and 19), and 40 (lanes 10 and 20) minutes. Lanes 1–10 and 11–20 were treated as two separate objects (corresponding to two different exposure times using traditional autoradiography) during PhosphorImager analysis to visualize RNA substrate and deadenylated product. Thus, the relative darkness caused by radioactivity in lanes 1–10 versus lanes 11–20 should not be compared visually.

FIG. T. The liberated reaction product during deadenylation is 5′-AMP. The 5′-AMP/7-methyl-GTP fraction was incubated with RNA substrate L3(A30), labeled by the inclusion of [32-P]GMP during in vitro transcription. A fraction of the reaction was subjected to two-dimensional TLC. The resulting autoradiogram of the dried polyethyleneimine-cellulose plate is shown. The location of 2′-AMP, 3′-AMP, and 5′-AMP markers are indicated.

A Functional Link between the RNA 5′ End Cap Structure and Poly(A) Tail Removal—The RNA substrate we have used during purification was capped at its 5′ end by the inclusion of m7G(5′)ppp(5′)G cap analogue during in vitro transcription of the L3(A30) RNA substrate. To investigate whether the presence of a cap at the 5′ end affected the nuclease activity of the 54-kDa fragment of PARN, we compared the relative specific activity of the nuclease using either capped or noncapped L3(A30) RNA, radioactively labeled in their homopolymeric tails, as the substrate. The released AMP was quantitated by the one-dimensional TLC assay. We found that the specific activity was approximately 6-fold higher using the capped L3(A30) RNA substrate compared with the noncapped RNA substrate, suggesting that a cap structure at the 5′ end stimulates the poly(A) tail removal activity of the 54-kDa fragment of PARN. To further investigate the role of the cap during poly(A) tail removal, we added in trans m7G(5′)ppp(5′)G, 5′ GMP or 5′-AMP to in vitro deadenylation reactions using capped L3(A30) RNA as the substrate. Fig. 8 shows that the free m7G(5′)ppp(5′)G cap analogue severely inhibited the deadenylation reaction already at 0.01 mM and completely inhibited the reaction at 0.1 mM, whereas 5′ GMP and 5′-AMP only inhibited the reaction at much higher concentration. The efficient inhibition of the nuclease activity by the cap analogue acting in trans and the stimulatory role of the cap acting in cis strongly suggest that the nuclease interacts with the cap structure during poly(A) degradation establishing a functional link between poly(A) tail removal at the 3′ end and the cap structure at the 5′ end of the RNA substrate.

DISCUSSION

In this paper we report on the purification to apparent homogeneity of a poly(A)-specific 3′ exonuclease activity (17, 18) (Figs. 1–3 and 5 and Tables I and II). A 54-kDa polypeptide (Fig. 3) corresponding to a fragment of the 74-kDa PARN nuclease (19, 20) copurified with the 3′ exonuclease activity. The molecular mass of the nuclease, as determined by gel filtration, was found to be 180–220 kDa (Fig. 4A). The purification of the poly(A)-specific 3′ exonuclease activity to apparent homogeneity is an important step toward mechanistic studies of the native nuclease. This work will therefore provide a solid platform for further studies using recombinant PARN nuclease as the source of activity.

The properties of the purified 54-kDa active fragment of
PARN were investigated and compared with a similar activity in HeLa cell free extracts that we previously have identified (17, 18). Both activities release 5′-AMP as the reaction product (Fig. 6), preferentially degrade poly(A) (Table II and Fig. 5A), and can only efficiently degrade poly(A) located at the 3′ end of the RNA substrate (Fig. 5B). Both activities generate a deadenylated RNA body during in vitro deadenylation (Fig. 5A), which upon prolonged incubation eventually will be degraded (data not shown). Both activities are strictly dependent on divalent cations, preferentially Mg2+, for their activities. The two activities have very similar chromatographic properties, and both can be purified by similar purification protocols (data not shown). We have not managed to obtain sufficient amount of the HeLa cell activity to unambiguously identify the 54-kDa PARN polypeptide by SDS-PAGE and silver staining. We conclude that the purified calf thymus poly(A) removing activity is a poly(A)-specific 3′ endonuclease and that it corresponds to the poly(A)-specific 3′ endonuclease that we previously identified in HeLa cell-free extracts.

The N- and C-terminal ends of the 54-kDa active fragment of PARN have not been unambiguously defined. However, we note that one of the peptides in the MALDI-TOF mass spectrum, 1461.69 in molecular weight, could correspond to the C-terminal end of the 54-kDa PARN fragment. Four lines of evidence support this suggestion: (i) a peptide of PARN consisting of amino acids 458–470 has the corresponding molecular weight; (ii) a lysine residue is located at amino acid 457 of PARN, which should be expected after digestion with trypsin; (iii) no peptide corresponding to predicted tryptic peptides located C-terminally of amino acid 471 of PARN was identified in the mass spectrum; and (iv) the calculated molecular mass of a predicted polypeptide consisting of amino acids 1–470 of PARN is 54.2 kDa, which corresponds to the molecular mass of the purified polypeptide, as determined by SDS-PAGE analysis. It is noteworthy that a tentative exonuclease domain belonging to the RNase D family of 3′-exonucleases (28, 37) is located within the N-terminally located 389 amino acids of human PARN (20).

We have investigated by Western blot analysis the presence of the 74-kDa PARN polypeptide in our fractions by using a polyclonal antibody directed against the C-terminal part of the 74-kDa PARN polypeptide (20) as the probe. Our data showed that large amount of the 74-kDa PARN polypeptide was present in the initial crude extract. However, the 74-kDa polypeptide did not copurify with the nuclease activity we selected based on our dual assay strategy.2 A very low abundant polypeptide in the size range of 80 kDa was visible in our purest fraction (Fig. 3). We have not been able to confirm that this polypeptide is related to the 74-kDa PARN polypeptide, neither by Western blot analysis or mass spectrometry. Unfortunately, it is not possible to investigate the presence of the 54-kDa polypeptide in the cruder fractions by Western blot analysis because of the inability of the antibody to recognize the 54-kDa PARN fragment. Thus, we cannot rule out the possibility that the 54-kDa active fragment of PARN was generated by proteolysis during purification. It is important to keep this possibility in mind when interpreting several of the properties of the nuclease activity that we have described in relation to the nuclease activity associated with the 74-kDa PARN polypeptide. However, high purity of an enzyme is a prerequisite for detailed mechanistic studies.

Körner et al. (20) detected two isoforms of PARN in Xenopus oocytes, one being 74 kDa in molecular size and the other 62 kDa. The 62-kDa form was the dominating form co-purifying with the nuclease activity after poly(A)-Sepharose chromatography. Interestingly, the two forms differed in subcellular distribution in Xenopus oocytes; the 62-kDa form being cytoplasmic, whereas the 74-kDa form was nuclear. It is therefore possible that the 54-kDa form that we purified represents an additional isoform of bovine PARN. Further experiments are required to resolve this issue (see also below).

The development of in vitro systems and the characterization of participating enzymes for polyadenylation and deadenylation will make it possible to reveal mechanisms regulating the poly(A) tail length. One interesting aspect is the competition between mRNA poly(A) tail addition and removal (reviewed in Refs. 38 and 39). The highly processive mode of degradation (Fig. 7) will definitely influence this competition, because a highly processive nuclease will compete very efficiently with the opposing poly(A) synthesis reaction once degradation has been initiated. This is in sharp contrast to a situation where a distributive nuclease activity will be competed by the poly(A) synthesis reaction after removal of each single adenosine residue.

An intriguing possibility could be that the processive mode of degradation is a unique property of the nuclease activity associated with the 54-kDa polypeptide of PARN because Körner et al. (20) previously reported that the 74-kDa PARN nuclease activity degraded poly(A) in a distributive fashion. Thus, the nuclease activity associated with the 74-kDa PARN polypeptide that they purified differed in one important mechanistic aspect compared with the 54-kDa PARN fragment activity. One reason for purifying two mechanistically distinct nuclease activities could be that different assay strategies were used to follow the nuclease activity during purification. Körner et al. (20) selected fractions for further purification based on the release of mononucleotides, whereas we selected fractions based on a dual assay strategy. This difference in assay strategies could very well be responsible for preferentially selecting

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2 J. Martinez, Y.-G. Ren, and A. Virtanen, unpublished observation.
a distributive nuclease activity in one case and a processive nuclease activity in the other case.

Three lines of evidence suggest an interaction between the nuclease associated with the 54-kDa active fragment of PARN and the mRNA cap structure: (i) the specific activity of the nuclease is higher for capped RNA substrate than for non-capped; (ii) a free cap analogue added in trans inhibits the nuclease activity (Fig. 8); and (iii) the nuclease activity interacts very strongly with the 7-methyl GTP matrix, which partly resembles a methylated cap structure. This matrix is frequently used to affinity purify cap binding proteins (40). All of this evidence suggests that the 54-kDa fragment of PARN contains a cap-binding site. The presence of a cap-binding site implies that the 54-kDa polypeptide interacts both with the 5' and the 3' end of the mRNA during deadenylation. We can only speculate about the functional importance of this interaction: (i) a physical interaction between the cap structure and the poly(A) tail during deadenylation will most likely interfere with the translational initiation process (reviewed in Ref. 41); thus, the interaction may be part of a signal that informs the translational machinery that the mRNA is subjected to degradation and should not be used for protein translation; (ii) the interaction between the cap and the poly(A) tail may play a role in defining a deadenylation-dependent decapping pathway of RNA degradation; (iii) the cap structure and the poly(A) tail may represent separate cis acting elements that together ensure that the PARN nuclease preferentially selects polyadenylated mRNA as the RNA substrate; and (iv) a physical interaction between the nuclease and the mRNA structure may stabilize the enzyme/substrate complex. The cap structure may therefore also play a role in making the 54-kDa PARN fragment a highly processive nuclease.

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Note added in Proof—Interaction between PARN and the cap structure has independently been observed by Gao et al. (Gao, M., Fritz, D. T., Ford, L. P., and Wilusz, J. (2000) Mol. Cell 5, 479–488) and Dehlin et al. (Dehlin, E., Wormington, M., Körner, C. G., and Wahle, E. (2000) EMBO J. 19, 1079–1086).

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