Purification and Characterization of a Polysome-associated Endoribonuclease That Degrades c-myc mRNA in Vitro*  
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The regulation of mRNA half-lives is determined by multiple factors, including the activity of the messenger RNases (mRNases) responsible for destroying mRNA molecules. Previously, we used cell-free mRNA decay assays to identify a polysome-associated endonuclease that cleaves c-myc mRNA within the coding region. A similar activity has been solubilized and partially purified from a high salt extract of adult rat liver polysomes. Based on a correlation between protein and enzyme activity, the endonuclease is tentatively identified as a ~39-kDa protein. It cleaves the coding region stability determinant of c-myc mRNA with considerable specificity. Cleavages occur predominantly in an A-rich segment of the RNA. The endonuclease is resistant to RNase A inhibitors, sensitive to vanadyl ribonucleoside complex, and dependent on magnesium. In these and other respects, the soluble enzyme we have purified resembles the polysome-associated c-myc mRNase.

This paper focuses on a ribosome-associated protein with the properties of a c-myc mRNA-degrading ribonuclease. The significance of mRNases stems from the fact that mRNA stability affects gene expression in virtually all organisms (1–4). Moreover, mRNA stability is regulated, because the half-lives of many mRNAs fluctuate as a function of cell growth, environmental factors, and the stage of cell differentiation. mRNases must play a central role in these fluctuations.

Unfortunately, we know little about mammalian mRNases, and no enzyme has yet been proved to be a mammalian mRNase. There are many unresolved issues about these enzymes. How many of them are present in each mammalian cell? In what critical ways do mRNases differ from RNA-processing enzymes? Do mRNases also process RNA or attack other classes of RNAs? How do mRNases distinguish mRNA from rRNA and tRNA? Does each mRNase attack only a subset of mRNAs? Genetic approaches have made it feasible to identify mRNases in lower organisms and to analyze the phenotype of cells with mRNA gene mutations (1, 2). In contrast, vertebrate cells with mRNase gene mutations have not been generated. Several candidate vertebrate mRNases have been identified using other strategies as follows: (i) incubating cell extracts with deproteinized mRNA substrates or (ii) analyzing mRNA decay in polysome-containing cell-free mRNA decay systems (reviewed in Refs. 3–5). Some of these enzymes are endonucleases, and others are exonucleases. Based on the findings in yeast and prokaryotes, it is likely that vertebrate cells contain only a few mRNases.

Our laboratory has been investigating the regulation of c-myc mRNA stability. By using cell-free mRNA decay assays and transfection, we and others (6) have suggested that c-myc mRNA can be degraded by alternative pathways involving at least two mRNases. One mRNase is involved in a 3’ to 5’ decay pathway in which the poly(A) tail is removed first, and then the body of the mRNA is degraded. The second mRNase is an endonuclease that we initially identified using a polysome-based cell-free mRNA decay assay. This endonuclease attacks the C-terminal coding region of endogenous, polysome-associated c-myc mRNA. We refer to this endonuclease cleavage target as the c-myc coding region determinant or CRD,1 because it is a major determinant of c-myc mRNA stability in cells (7–12). It is also a binding site for a protein that is thought to shield the mRNA from endonuclease attack (9, 10, 13, 14).2 The relationship of the endonuclease to the c-myc CRD and to c-myc mRNA stability is further supported by the finding that c-myc mRNA can be degraded endonucleolytically in cells (16, 17).

Here, we describe the partial purification of a polysome-associated endonuclease with the properties of the c-myc mRNase. The soluble enzyme was isolated from a high salt extract of rat liver polysomes. It is a magnesium-dependent protein that is resistant to the RNase A class of RNase inhibitors. Therefore, it is not a member of the RNase A family. It cleaves deproteinized c-myc CRD RNA with high specificity and preferentially attacks one RNA segment that is rich in A residues. Although these data do not prove that this enzyme is the c-myc mRNase, the enzyme we have purified does share several properties with the polysome-associated c-myc mRNase.

**EXPERIMENTAL PROCEDURES**

**Preparation of Radiolabeled Transcripts**—The subcloning of the c-myc DNA fragments corresponding to all or part of the CRD of c-myc mRNA has been described (9, 13). Transcription of these DNAs generates RNAs corresponding to nucleotides 1705–1762 and 1705–1886 of c-myc mRNA, respectively (diagrammed in Fig. 1A, top). The 1705–1886 RNA is the full-length CRD and is designated FL-CRD RNA in the text. The shielding protein or CRD-binding protein (CRD-BP) binds with high specificity to FL-CRD RNA (10, 13). The RNA corresponding to c-myc nts 1705–1792 is referred to as 5’-CRD RNA. 5’-CRD 32P-RNA was used as the substrate for purifying and characterizing the liver endonuclease. Unlabeled CRD RNA was synthesized by linearizing the 5’-CRD plasmid or the FL-CRD plasmid with EcoRI and transcribing

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the DNA with SP6 RNA polymerase. As a control, plasmid pBSmyc, which contains full-length human c-MYC cDNA cloned into pBlue-script, was digested with Ear-I and transcribed with T7 RNA polymerase to generate a RNA corresponding to nucleotides 1–217 of c-MYC mRNA. Transcriptions were performed using Megascript kits (Ambion). Each transcription was resuspended in 10 µl 10 mM Tris-Cl, pH 7.6. These components were first assembled on ice, before being layered over a 10-ml cushion of 30% (w/v) sucrose in Buffer A and centrifuged in the SW28 rotor at 96,500 g for 10 min. The post-nuclear supernatant was layered over a 10-ml cushion of 25% (v/v) glycerol. Three hundred mg of liver RSW protein at 37 °C for at least 6 months without loss of endonuclease activity.

Purification of the Solubilized Liver Polysomal Endonuclease—All procedures were performed at 4 °C. Approximately 4 g of RSW protein were mixed with 6 volumes of 25% (v/v) glycerol. Three hundred mg of RSW protein were obtained from one liver. RSW can be stored at -80 °C for at least 6 months without loss of endonuclease activity.

Preparation of Ribosomal Salt Wash (RSW) from Rat Liver—All procedures were performed at 4 °C. Seventy male Sprague-Dawley rats (Harlan Sprague-Dawley) each weighing 175–200 g were sacrificed. Their livers (average weight 11 g) were excised. One ml of Buffer A per (Harlan Sprague-Dawley) each weighing 175–200 g were sacrificed.

endpoint was the radioactive decay product of the 88-nt CRD RNA was incubated in a 25-µl reaction containing 90 pmol of [5-32P]pCp (Amersham Pharmacia Biotech, 3000 Ci/mmol). 10 mM MgCl₂, 5 mM DTT, 200 ng of BSA, 10% (v/v) Me₂SO, 1 mM ATP, 40 units of RNasin (Promega), 120 units of T4 RNA ligase (New England Biolabs), 50 mM HEPES, pH 8.3, for 18–20 h at 4 °C. The resulting RNA was 3'-end-labeled and 3'-phosphorylated (RNA-32P-C-P). Unincorporated label was removed, and the RNA was purified as described above. In Vitro Assay for Soluble Liver Endonuclease Activity—The pH of all buffers for these experiments was determined at room temperature. The standard 20-µl reaction mixture included 2 mM DTT, 1 unit of RNasin (Promega), 2 mM magnesium acetate, 50 mM potassium acetate, 0.1 mM spermidine, 1 ng of 5'-end-labeled [32P]-RNA (~5 × 10⁶ cpm), 11 mM Tris-HCl, pH 7.6. These components were first assembled on ice, and enzyme was added last. Unless otherwise noted, reactions were incubated for 10 min at 37 °C, placed in dry ice for 10 min, and then lysophosphatidylcholine. Five µl of loading dye (80% [v/v] formamide, 0.1% xylene cyanol, 12.5 mM EDTA, pH 8.0) were added, and the samples were denatured at 65 °C for 10 min and electrophoresed at 250 V for 2 h in an 8% polyacrylamide, 7 M urea gel. Gels were fixed in 10% acetic acid, 10% methanol for 15 min, dried, and exposed to a PhosphorImager screen (Molecular Dynamics). One major endonuclease decay product of ~30 nt was generated (see Fig. 1D). One endonuclease unit was defined as the amount of enzyme required to cleave 30% of the input RNA substrate into this decay product in a 10-min reaction at 37 °C. Under these conditions, the amount of decay product formed was linear with enzyme concentration.

FIG. 1. c-myc CRD RNA-degrading endonuclease activity in rat liver ribosomal salt wash. A, top, endonuclease RNA substrate. Human c-MYC mRNA is diagrammed at the top, with its CRD indicated by the hatched box. In the text, this 182 c-myc RNA segment is designated FL-CRD. FL-CRD RNA corresponds to c-myc nucleotides 1705–1886. The 88-nt RNA corresponding to c-myc nts 1705–1792 is from the 5’-half of FL-CRD RNA. In the text, it is designated 5’-CRD. Unless otherwise noted, 5’-CRD RNA 32P-labeled at its 5’-end was the substrate for all liver endonuclease assays. A, bottom, diagram of GMG mRNA. The gene encoding GMG mRNA is described under “Experimental Procedures” and in Herrick and Ross (10). GMG mRNA consists of the FL-CRD of human c-MYC RNA plus an additional 67 c-myc coding nts subcloned in frame into human β-globin. GMG mRNA was expressed in HeLa cells and was used as an endogenous, polysome-bound mRNA substrate to assay the polysome-associated c-myc mRNAase. B, endonuclease activity in crude rat liver RSW. 5’-CRD 32P-RNA was incubated with or without 10 µg of liver RSW protein at 37 °C for the indicated times. RNA was extracted and electrophoresed in an 8% polyacrylamide, 7 M urea gel. Unfilled arrowhead, undigested 5’-CRD RNA. Filled arrowhead, major endonuclease decay product, which migrates at 30–40 nts. In gels with greater resolving power, this band migrates at ~30 nt (Fig. 6). The marker is 5’-end-labeled pBR322 32P-DNA cleaved with HaeII. The length of each marker fragment is noted in nts on the left.
ity of all other fractions was calculated (Fig. 2).

Pooled phosphocellulose fractions (Fig. 2A) were diluted to 0.1 M KCl by adding 1 volume of 20% (v/v) glycerol, 2 mM DTT, 60 mM triethanolamine, pH 7.4. The sample was applied at a flow rate of 1 ml/min to a 2.5 × 16 cm reactive blue-3 dye affinity column (Sigma) equilibrated with 0.1 M KCl, 2 mM DTT, 10% (v/v) glycerol, 20 mM triethanolamine, pH 7.4 (Buffer B). After washing the column until the A<sub>280</sub> returned to baseline, proteins were eluted with a linear gradient from 0.1 to 1.0 M KCl in Buffer B. Three separate reactive blue-3 columns were performed, and similar results were obtained from each.

Active fractions from reactive blue-3 columns were diluted to 0.1 M KCl by adding 1 volume of 25% (v/v) glycerol. The sample was applied at a flow rate of 1.5 ml/min onto 1.2 × 2.0 cm Q-Sepharose (Amersham Pharmacia Biotech) column equilibrated with Buffer B. The flow-through containing the RNase activity was collected until a baseline A<sub>280</sub> was reached. To ensure that no RNase remained on the column, the column was washed with buffer containing 1 M KCl to elute bound proteins. No RNase activity was detected in this wash (data not shown).

The Q-Sepharose flow-through was loaded at a flow rate of 1.2 ml/min onto a 2.5 × 4.0 cm reactive green-19 column (Sigma) previously equilibrated with Buffer B. After washing until the A<sub>280</sub> returned to base line, bound proteins were eluted with a linear gradient from 0.1 to 1.0 M KCl in Buffer B. Three-ml fractions were collected into tubes containing 150 μg of carrier carbonic anhydrase (Sigma), which was included from this stage on at a final concentration of 100 μg/ml to maintain endonuclease activity.

Active fractions from reactive green-19 were pooled and diluted to 0.1 M KCl by adding 5 volumes of 25% (v/v) glycerol containing 100 μg/ml carbonic anhydrase. The sample was loaded at a flow rate of 1.2 ml/min onto a pre-packed 5 ml heparin-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 0.1 M KCl, 2 mM DTT, 10% (v/v) glycerol, 25 mM potassium phosphate, pH 7.4. The column was washed until the A<sub>280</sub> returned to baseline, and bound proteins were eluted with a linear gradient from 0.1 to 1.0 M KCl in the same buffer. Fractions of 1.4 ml were collected into tubes containing 100 μg of carrier carbonic anhydrase.

Glycerol Gradient Centrifugation of Liver Ribosomal Salt Wash (RSW)—RSW (0.1 ml; 1 mg of protein) was layered onto a 10–30% (v/v) glycerol gradient (4 ml, 11 × 60-mm tubes) made in 0.25 M KCl, 0.1 mM EDTA, 50 mM Tris-HCl, pH 7.4, and was centrifuged for 18 h, 4 °C, 200,000 × g (44, 100 rpm) in a Beckman SW 60 rotor. Fractions of 0.2 ml were collected manually from the top, and 5 μl of each fraction were assayed for endonuclease activity. A separate 10–30% glycerol gradient was centrifuged in the same manner and contained the following proteins as molecular mass standards: β-galactosidase (116.4 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.3 kDa).
The existence of a polysomal mRNA-degrading endonuclease was revealed in previous studies using a cell-free mRNA decay assay (9). The assay reaction includes polysomes from tissue culture cells, and the decay of endogenous, polysome-associated mRNAs such as c-myc mRNA is cleaved endonucleolytically in a c-myc segment designated the coding region determinant or CRD. The CRD is the last 180–250 nt of the mRNA 3′ terminus. The c-myc region of human β-globin CDNA prepared by random priming. This probe anneals to the 5′ region of GMG mRNA and recognizes both undegraded GMG mRNA and the 5′-endonuclease decay product.

Mapping RNA 3′ Ends—The method of Zaug et al. (19) was used, with slight modifications. Eight ng of 5′-32P-labeled 5′-CRD RNA (c-myc nucleotides 1705–1792) were cleaved by 1 unit of heparin-Sepharose-purified endonuclease. The reaction was terminated at several time points, and the RNA was extracted with phenol/chloroform. A 1.5-ng aliquot of each RNA sample was electrophoresed in an 8% denaturing polyacrylamide gel to confirm that the endonuclease had cleaved the RNA. The remainder of each sample (6.5 ng) was poly(A)-tailed with yeast poly(A) polymerase (550 units; U. S. Biochemical Corp.) in a 20-μl reaction according to the manufacturer’s instructions. The poly(A)-tailed RNA was then reverse-transcribed with 20 units of avian myeloblastosis virus-reverse transcriptase (Boehringer Mannheim) using 5 μg of primer T (5′-dAACCCGGCTCGAGCGGCCGC(T18)-3′) in a 20-μl reaction containing 8 mM MgCl2, 30 mM KCl, 1 mM DTT, 1 mM each of the dNTPs, 4 units of RNAsin, 50 mM Tris-HCl, pH 8.5. The underlined nucleotides in the primer T sequence denote the XhoI restriction site used for cloning. The reaction was incubated first at room temperature for 15 min and then at 45 °C for 15 min. Reverse transcriptase was inactivated by heating the reaction mix to 95 °C for 5 min, and unincorporated dNTPs were removed with a 50–5 spin column (American Pharmacia Biotech). Amplification by PCR was performed using primers T and Myc. The Myc primer (5′-dTCGGATCCATTTAGGT-9GACACTATAGAGCAGCTGGTTG-3′) includes c-myc nucleotides 1705–1722. The underlined nucleotides indicate a BamHI restriction site. Two DNAs were detected following PCR. One corresponded to undegraded 5′-CRD RNA and the other to the endonucleolytic degradation product (see Fig. 10B). Each DNA was gel-purified, cleaved with XhoI and BamHI, cloned into pGEM7Z, and sequenced using a T7 primer.

RESULTS

Identification and Purification of a Polysomal, c-myc mRNA-degrading Endonuclease—The existence of a polysomal c-myc mRNA-degrading endonuclease was revealed in previous studies using a cell-free mRNA decay assay (9). The assay reaction includes polysomes from tissue culture cells, and the decay of endogenous, polysome-associated mRNAs such as c-myc and histone is monitored. Under certain reaction conditions, polysome-associated c-myc mRNA is cleaved endonucleolytically in a c-myc segment designated the coding region determinant or CRD. The CRD is the last 180–250 nt of the

gene is driven by the cytomegalovirus immediate-early promoter, and the major features of GMG mRNA are diagrammed in Fig. 1A, bottom. GMG mRNA includes 419 nt from the human β-globin mRNA cap site to the EcoRI site in the coding region, 249 nt from the C-terminal coding region of human c-myc mRNA, 6 nt from an EcoRI linker, and 207 nt from the β-globin EcoRI site to the mRNA 3′ terminus. The c-myc segment is inserted in frame and includes the 182-nt c-myc CRD plus 67 coding nt of the CRD, mRNA decay reactions contained polysomes from GMG-expressing HeLa cells and were performed as described previously (9, 10, 18). Where indicated, excess c-myc CRD competitor RNA was added to the reactions at 1 μg of competitor per 10 μg of polysomal RNA. The CRD competitor RNA activates the c-myc endonucleolytic decay pathway and causes the mRNA to be cleaved within the CRD (9). After incubation at 37 °C for various times, total RNA was prepared by phenol extraction and was blotted to a Hybond-N+ membrane (Amersham Pharmacia Biotech). Blots were hybridized with a [32P]ApsLI-EcoRI fragment of human β-globin CDNA prepared by random priming. This probe anneals to the 5′ region of GMG mRNA and recognizes both undegraded GMG mRNA and the 5′-endonuclease decay product.

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coding region (Fig. 1A; Ref. 9). Two additional findings strengthened the connections among an endonuclease, the CRD of c-myc mRNA, and c-myc mRNA stability. (i) The CRD is a major determinant of c-myc mRNA expression and stability in vivo (7, 8, 10–12). (ii) c-myc mRNA is cleaved endonucleolytically in at least some cells (16, 17). In view of these findings, we undertook to purify and characterize the responsible enzyme. The strategy was to solubilize polysomal proteins using high salt extraction to incubate the extract with a deproteinized c-myc CRD 32P-RNA substrate. Since endonucleolytic cleavage of endogenous c-myc mRNA occurred in the 5'-one-half of the CRD (9, 10, 13), RNA from this segment of the CRD was used as substrate (designated 5'-CRD RNA; Fig. 1A).

A high salt ribosomal salt wash extract (RSW) from rat liver polysomes was chosen as the starting material for these studies for three reasons. (i) c-myc mRNA abundance is regulated post-transcriptionally in rodent liver cells during fetal development and during liver regeneration (20, 21). The c-myc coding region is required for proper regulation to occur (22–26). Therefore, it seemed reasonable to search for an endonucleolytic c-myc mRNAse in liver tissue. (ii) Liver provides an abundant source of starting material. (iii) Crude liver extract contains an endonuclease with the expected properties of the c-myc mRNAse. When c-myc 5'-CRD 32P-RNA was incubated with rat liver RSW, the RNA was cleaved endonucleolytically, generating a single major degradation product of ~30–40 nts (Fig. 1B,

**Fig. 4. Chromatography of the liver endonuclease on heparin-Sepharose.** A, SDS-PAGE of heparin-Sepharose column fractions. Thirty-μl aliquots from fractions 14–23 of the heparin-Sepharose column (Fig. 2E) were separated by 12% SDS-PAGE, and proteins were visualized by silver staining. All fractions contained carrier carbonic anhydrase to maintain endonuclease activity. Marker, low molecular mass standards (Bio-Rad); molecular mass is indicated in kDa on the left. Arrow, the ~39-kDa band that comigrates with endonuclease activity. B, endonuclease activity in heparin-Sepharose fractions. A 1-μl aliquot of fractions 14–23 from heparin-Sepharose was assayed for endonuclease activity by incubation with 5'-CRD 32P-RNA at 37 °C for 30 s or for 2 min, as indicated. RNA cleavage was determined by denaturing gel electrophoresis. Unfilled arrowhead, undigested RNA; filled arrowhead, major endonucleolytic decay product.
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FIG. 5. Analysis of the liver endonuclease by size-exclusion chromatography and glycerol gradient centrifugation. Filled circles, endonuclease activity measured as percent of maximal endonucleolytic activity. Open circles, low molecular mass standards (Bio-Rad). A, SEC 2000 size exclusion high-pressure liquid chromatography. The column was calibrated with molecular mass markers before chromatography of 4 mg of rat liver RSW. B, glycerol gradient centrifugation. One mg of rat liver RSW was centrifuged in a 10–30% glycerol gradient. A parallel gradient contained molecular mass marker proteins. The enzyme did not bind to Q-Sepharose at 0.1 M salt.

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endonuclease lost activity when the protein concentration fell below 50 μg/ml (data not shown), carrier carbonic anhydrase was added to all fractions in the final two purification steps (reactive green-19 and heparin-Sepharose). As a result, endonuclease activity was maintained, but it became impossible to determine the fold purification of the enzyme (Table I). The endonuclease activity recovered from the heparin-Sepharose column contained many proteins (Fig. 3). However, a comparison of enzyme activity with the protein elution profile suggested that the endonuclease corresponded to the ~39-kDa protein denoted by the arrow in Fig. 3. The major peak of endonuclease activity was detected in heparin-Sepharose fractions 17–19; there was also considerable activity in fractions 20–22 (Fig. 4B). Of all the proteins in these fractions, the only one whose intensity correlated with enzyme activity was the ~39-kDa band (Fig. 4A, arrow). Note also that some of the other bands in these lanes were derived from the added carrier protein.

Two additional experiments implicated the ~39-kDa band as the endonuclease. First, size-exclusion chromatography was performed on liver RSW, and two endonuclease peaks were observed, one at ~40 kDa the other at ~80 kDa (Fig. 5A). Second, glycerol gradient centrifugation was performed. Three peaks of endonuclease activity were observed, a prominent, broad one centered at ~35 kDa and lesser peaks at ~85 and ~110 kDa (Fig. 5B). We have not determined whether the 85- and 110-kDa activity peaks represent distinct enzymes or multimeric complexes containing the ~39-kDa endonuclease. It would not be surprising if the endonuclease formed multimers with itself or with other proteins, because dimer/multimer formation is a common feature of many RNases (27–30).

The Enzyme Is an Endonuclease—To prove that the enzyme is an endonuclease, it was incubated with 88-nt CRD RNA that was 32P-labeled at either the 5’ or 3’ terminus. 5’-Labeled substrate was cleaved primarily at a single site located ~30 nt 3’ of its 5’ terminus (Fig. 6, left 3 lanes). 3’-Labeled substrate was also cleaved at a single major site located ~60 nt from its 3’ terminus (Fig. 6, lanes denoted ‘3’ Label). Therefore, the combined sizes of these cleavage products correspond to the size of the full-length substrate. Moreover, in this and other experiments, the amounts of radioactivity in each cleavage product plus the remaining full-length substrate were equal to the amount of substrate added at time 0 (data not shown). This result further confirms that a single cleavage event generates the two cleavage products. In summary, the enzyme is an endonuclease.

Properties of the Purified Polysomal Endonuclease—The heparin-Sepharose-purified endonuclease was active between 0 and 100 mM KCl but lost activity at 200 mM or higher KCl (Fig. 7A). It remained active when pre-heated to 42 °C but was completely inactivated at 50 °C (Fig. 7B). It did not require a nucleic acid cofactor, because its activity was unaffected by pretreatment with micrococcal nuclease (60 units; data not shown). It was completely inactivated by proteinase K treatment (0.02 mg/ml; data not shown).

Similarities between the Soluble Rat Liver Endonuclease and the Polysome-associated c-myc mRNAse—We have exploited two assays to identify a c-myc mRNA-degrading endonuclease. (i) Polysomes from cultured cells are incubated under conditions that activate the endonuclease (see below). This enzyme is designated the polysomal c-myc mRNAse. (ii) Solubilized enzyme from liver polysomes is incubated with deproteinized c-myc CRD 32P-RNA (Fig. 1B). The goal of the experiments described below was to compare several properties of the polysomal c-myc mRNAse with the solubilized liver enzyme.

As noted above, endogenous, polysome-associated c-myc...
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Fig. 6. The polysomal enzyme is an endonuclease. The 88-nt CRD RNA substrate was 32P-labeled at its 5′ or its 3′ terminus. Each RNA was then incubated under standard conditions for the indicated times, when the reactions were stopped and the RNA was harvested. Each RNA was electrophoresed, and the bands were visualized by autoradiography. They were also quantified by PhosphImager. Lane M, 5′-end-labeled pBR322 32P-DNA cleaved with HaeIII. The marker bands from top to bottom are 83, 60, and 53 nt, respectively.

mRNA can be degraded in vitro by either of two alternative pathways, one of which is endonucleolytic. In order to activate the endonucleolytic pathway, the reactions are supplemented with excess competitor RNA corresponding to the c-myc FL-CRD (Fig. 1A, top). The activation of endonucleolytic decay by competitor FL-CRD RNA led to a model suggesting that the c-myc decay pathway was determined by whether or not a CRD-binding protein (CRD-BP) was bound to c-myc mRNA (9, 10, 13, 14). The model is summarized as follows. When the CRD-BP is bound to polysome-associated c-myc mRNA, the CRD-BP shields the mRNA from the endonuclease. The mRNA is still unstable but is destroyed in a 3′ to 5′ direction (6). When excess FL-CRD competitor RNA is added, the CRD-BP dissociates from c-myc mRNA, deprotecting the CRD and making the CRD susceptible to endonuclease attack. As a result, c-myc mRNA is cleaved within its CRD by the polysome-associated endonuclease, generating discrete endonucleolytic decay products. Consistent with this model, we have purified and cloned the c-myc CRD-BP (13).

The liver endonuclease we have purified was initially recognized as a candidate c-myc mRNase because it and the polysome-bound c-myc mRNase both cleave at or near the same site in the c-myc mRNA CRD. The following experiments were performed to compare additional properties between the soluble liver enzyme and the polysomal c-myc mRNase. The polysomal mRNase was assayed by incubating polysomes with excess, unlabeled c-myc FL-CRD RNA and analyzing the c-myc endonucleolytic decay product by Northern blotting. The polysomes were prepared from HeLa cells expressing a chimeric protein that consists of the CRD region of c-myc mRNA indicated by filled arrowhead; undegraded GMG mRNA indicated by unfilled arrowhead). The cleavage site was within the c-myc CRD segment of GMG mRNA (9, 10). These data are significant, because they indicate that the polysomal c-myc mRNase and the liver endonuclease are not members of the RNase A family of RNases. The effect of vanadyl ribonucleoside complex (VRC) was also tested on both RNases. The mechanism of action of VRC differs from that of the RNase A inhibitors, and VRC is known to inhibit some but not all RNases (31). VRC completely inhibited the purified liver endonuclease (Fig. 8A, lanes 10–13). It also inhibited the polysome-associated c-myc mRNase (Fig. 9A, last 4 lanes on the right). The soluble liver endonuclease and the polysome-associated c-myc mRNase are also both magnesium-dependent (Figs. 8B and 9B). In the absence of magnesium, neither enzyme was active, whereas both enzymes were active in magnesium concentrations from 1 to 10 mM. In summary, the liver endonuclease and the polysome-associated c-myc mRNase share several properties. Both attack the CRD segment of c-myc mRNA, are insensitive to RNasin, are sensitive to VRC, and are dependent on divalent cation (magnesium).

Substrate Specificity of the Purified Polysomal Endonuclease—We challenged the purified endonuclease with three deproteinized 5′-32P-RNA substrates as follows: c-myc 5′-CRD RNA (nts 1705–1792), c-myc FL-CRD RNA (nts 1705–1886), and nts 1–217 from the c-myc mRNA 5′-UTR. The following observations were made (Fig. 10). (i) Both CRD RNAs were attacked in the same region and generated the same prominent decay product (Fig. 10, asterisk). No other prominent CRD RNA decay products were detected. (ii) A discrete decay product was not observed with c-myc 5′-UTR (nts 1–217) RNA. However, this RNA was degraded by the liver enzyme, as determined by quantitating the amount of full-length (nts 1–217) RNA destroyed by the enzyme (Fig. 10, numbers at bottom). Approximately 49, 57, and 37% of the 5′-CRD, FL-CRD, and (nts 1–217) RNAs were degraded, respectively. Either the (nts 1–217) RNA was not cleaved in a single region or was cleaved so close to its 5′ terminus that the resulting 5′ decay product was electrophoresed off of the gel. In either case, these data indicate that...
the purified endonuclease cleaves other deproteinized RNAs besides c-myc CRD RNA. Moreover, when present in excess, it probably cleaves CRD RNA at sites other than the major one (for example, see results with fraction 18, Fig. 4B, 2-min reaction). Therefore, the liver endonuclease not is a “restriction RNase” in the sense that it cleaves deproteinized RNA at only one specific site or that it cleaves only one RNA substrate. On the other hand, it does exhibit considerable specificity. When challenged with the 182-nt FL-CRD RNA substrate, it clearly prefers to cleave in one region (Fig. 10).

Mapping the Major Endonuclease Cleavage Site within c-myc CRD RNA—A PCR-based method was exploited to map precisely the endonuclease cleavage site within the 5'-CRD 32P-RNA (19). First, the RNA was incubated with heparin-Sepharose-purified endonuclease between 12 s and 10 min. During this time the amount of the expected decay product increased (Fig. 11A, filled arrowhead), and the substrate RNA decreased (unfilled arrowhead). An aliquot from each time point was poly(A)-tailed using yeast poly(A) polymerase. cDNA was prepared by reverse transcribing the tagged RNA using an oligo(dT) primer. Then, the cDNA was amplified by PCR, and PCR products were visualized following electrophoresis in a 2% agarose gel (Fig. 11B). As expected, no PCR product was detected with non-tailed RNA (Fig. 11B, lane 2). An -170-bp band was observed with poly(A)-tailed RNA that was not incubated with the endonuclease (Fig. 11B, lanes 3 and 4, unfilled arrowhead). The -170-bp DNA corresponds to the major endonucleolytic decay product. To map each PCR product, the large and small bands from the 5-min time point of Fig. 11B were excised, gel-purified, subcloned, and sequenced. Eight clones from the large DNA and 25 clones from the small DNA were sequenced, and the data are summarized in Fig. 12. The 3' terminus of all eight large DNA clones corresponded to the 3' end of the original RNA substrate, which was c-myc nt 1792 (Fig. 12A). There was some variation in the downstream sequence, and one of the 8 clones lacked the expected EcoRI site used for subcloning. Nevertheless, the data clearly validate the assay and confirm that the large band of Fig. 11B was derived from undegraded DNA.
GMG mRNA and the 5' transfectants were selected (10). The GMG gene is driven by a cytomegalovirus promoter and generates GMG mRNA, which is diagrammed in Fig. 1. Polysomes were made from this cell line and were incubated in the presence or absence of 1 μg of competitor c-myc CRD RNA. The competitor RNA activates the endonucleolytic decay pathway for c-myc and GMG mRNAs, and the responsible enzyme is designated the polysome-associated c-myc mRNase (9, 10). Reactions were incubated for the indicated times. Total RNA was isolated, electrophoresed, and analyzed by Northern blotting using a human β-globin 32P-DNA probe that anneals to the 5' region of GMG mRNA. Therefore, this probe recognizes both undegraded GMG mRNA and the 5'-endonuclease decay product. Unfilled arrowheads, undegraded GMG mRNA; filled arrowheads, the 5' decay product resulting from endonucleolytic cleavage of GMG mRNA within its c-myc CRD segment. RNA molecular weight markers are indicated in kilobases on the left. A, effect of RNase inhibitors. Polysomes were incubated in reactions containing either 1 unit of RNasin or 20 mM VRC. Some reactions were supplemented with CRD competitor RNA, as noted, to activate the endonucleolytic decay pathway. B, magnesium requirement for endonucleolytic cleavage. In the top lanes, the reactions contained no magnesium acetate but did contain 10 mM EDTA. In the remaining lanes, reactions did not contain EDTA but did contain the indicated amount of magnesium acetate and, where noted, competitor CRD RNA.

DISCUSSION

Three convergent observations highlight the significance of the liver endonuclease and its potential role as a c-myc mRNase. (a) The CRD of c-myc mRNA is cleaved in a specific region by the liver endonuclease. The c-myc CRD is also an instability determinant and potential endonuclease target site in cells, as indicated by the following findings. (i) c-myc mRNA is unstable even when its 5'- and 3'-UTRs are both deleted (32–34). Therefore, the c-myc coding region contains an instability determinant. (ii) The c-myc CRD is required for down-regulating c-myc mRNA post-transcriptionally when myoblasts fuse to form multinucleated myotubes (7, 8, 11, 12). Down-regulation does not occur when the CRD is deleted. (iii) The coding region is also required to regulate c-myc mRNA post-transcriptionally in liver cells. c-myc mRNA levels increase transiently and then decrease during the liver regeneration process that occurs following partial hepatectomy in adult rodents (20, 21). These fluctuations in c-myc mRNA abundance are regulated post-transcriptionally and are dependent on the c-myc mRNA coding region, not the c-myc gene promoter (22–25). (iv) The CRD is an mRNA instability element in cells independent of other c-myc mRNA regions. When the c-myc CRD is inserted into the coding region of globin mRNA to generate globin-MYC-globin mRNA (Fig. 1A, bottom), this mRNA is destabilized in cells (10). (b) In cells, c-myc mRNA is degraded by alternative pathways, one of which is endonucleolytic. Like many other mRNAs containing AU-rich elements in their 3'-UTRs, c-myc mRNA can be degraded 3' to 5' in cells (16, 35). However, it can also be degraded endonucleolytically (16, 17). (c) Under appropriate conditions in polysome-based
cell-free mRNA decay assays, c-myc mRNA is degraded by endonucleolytic cleavage within its CRD. The responsible endonuclease is associated with polysomes, because polysomes are the only cellular constituents in the assay mix (9).

Based on these findings, we sought to solubilize and characterize an endonuclease whose properties reflect those of the polysome-associated c-myc mRNase. The soluble liver enzyme, like the polysomal mRNase, is insensitive to RNasin, sensitive to VRC, and dependent on magnesium (Figs. 8 and 9). We have not proved that the purified enzyme is the only liver polysomal endonuclease with these properties. However, it is clear that the RSW from which the purification began does not contain multiple RNasin-insensitive endonucleases (Figs. 2).

Two findings suggest that the soluble endonuclease might be the ~39-kDa protein band marked by the arrow in Fig. 3. (i) The presence of this band correlates with endonuclease activity in heparin-Sepharose fractions (Fig. 4). (ii) The endonuclease migrates in the 35–40-kDa range by size exclusion chromatography and in a glycerol gradient (Fig. 5). It might be significant that higher molecular weight endonuclease activities were also detected in these assays. Perhaps this endonuclease, like other RNases, multimerizes or associates with other proteins (27–30).

The substrate specificity of the liver enzyme is not absolute but is nevertheless striking. Cleavage occurs in one major region of deproteinized 5'-CRD RNA (Figs. 6 and 12). The same region is also preferentially cleaved when FL-CRD RNA is the substrate (Fig. 10). This region is A-rich, and cleavage might occur at the A residues themselves. If so, it is unclear why nts 1727–1736 are preferentially cleaved, whereas other A-rich or purine-rich sites are spared (for example, nts 1758–1763). Two other sites in FL-CRD RNA (nts 1801–1804 and 1833–1835) are also A-rich but are not cleaved preferentially by the liver endonuclease. Perhaps the enzyme recognizes primary sequence and secondary structure, thereby limiting its access to most sites, even A-rich ones. We have postulated that unique features of the c-myc CRD make it an avid binding site for a shielding protein, the CRD-BP (9, 13). If so, it would be of interest to determine whether mutant CRD RNAs that bind poorly to the CRD-BP are also poor substrates for the endonuclease.

To our knowledge, the polysomal endonuclease is a novel enzyme. Several other vertebrate endonucleases have been reported by other labs. These include a 13.3-kDa human endonuclease that shares several core functional properties with the E. coli enzyme RNase E (36), a 65-kDa enzyme that also shares some properties with E. coli RNase E (37), an ~60-kDa Xenopus liver polysomal endonuclease that preferentially attacks albumin mRNA (38, 39), an ~120-kDa endonuclease from Xenopus and Drosophila cells that preferentially attacks maternal homeobox mRNAs (40, 41), and an ~68-kDa endonuclease from human T cells that preferentially attacks interleukin-2 mRNA in vitro (15). The endonuclease we have purified differs in at least one respect from all of these enzymes. None of these enzymes, including the one reported here, has yet been proved conclusively to be mRNase. This is unfortunate, because we will not fully understand how vertebrate mRNAs are degraded until we identify vertebrate mRNases (3, 4). By having par-

![Image](https://example.com/image.png)

**Fig. 10. Substrate specificity of the purified liver endonuclease.** One unit of heparin-Sepharose-purified liver endonuclease was incubated for 10 min at 37 °C with the following deproteinized, 5'-end-labeled 32P-RNAs: 5'-CRD (c-myc nts 1705–1792), FL-CRD (c-myc nts 1705–1886), and RNA corresponding to nts 1–217 from the 5'-UTR of c-myc mRNA. Asterisks indicate the cleavage product generated from 5'-CRD or FL-CRD RNA. Numbers at the bottom indicate the percentage of input RNA that was degraded. These numbers were obtained using the PhosphorImager to quantitate the amount of undegraded RNA in reactions without and with endonuclease. Marker, 5'-end-labeled pBR322 32P-DNA cleaved with HaeIII; sizes are noted in nts on the left.

![Image](https://example.com/image.png)

**Fig. 11. PCR amplification of c-myc 5'-CRD RNA that was cleaved by purified liver endonuclease.** One unit of heparin-Sepharose-purified endonuclease was incubated with 5'-end-labeled 5'-CRD 32P-RNA under standard conditions for the indicated times. A, time course. RNA was harvested at the indicated times, and one portion was electrophoresed in an 8% polyacrylamide, 7 M urea gel. Unfilled arrowhead, undegraded RNA substrate; filled arrowhead, endonucleolytic cleavage product. Marker, 5'-end-labeled pBR322 32P-DNA cleaved with HaeIII; sizes are noted in nts on the left. B, PCR amplification of the endonucleolytic cleavage product. An aliquot of total RNA from the reactions described in A was tailed with poly(A) and reverse-transcribed. The resulting cDNAs were then amplified by PCR (“Experimental Procedures”). The PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide. Unfilled arrow, PCR product corresponding to undegraded 5'-CRD RNA; filled arrow, PCR product corresponding to the endonucleolytic cleavage product. Each PCR product was further characterized by sequencing as per Fig. 12. In Fig. 12, the PCR products corresponding to the unfilled and filled arrowheads are designated Large and Small, respectively.
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Sepharose-purified liver endonuclease as per Fig. 11. A and amplified by PCR. Two major products were observed and were added enzymatically to the RNA, which was then reverse-transcribed.

how the enzyme affects mRNA stability in cells.

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