Identification of in Vivo mRNA Decay Intermediates Corresponding to Sites of in Vitro Cleavage by Polysomal Ribonuclease 1*

Mark N. Hanson‡§ and Daniel R. Schoenberg‡¶
From the ‡Department of Molecular and Cellular Biochemistry, The Comprehensive Cancer Center, and the ¶Molecular, Cellular, and Developmental Biology Graduate Program, The Ohio State University, Columbus, Ohio 43210

Previous work from this laboratory identified a polysome-associated endonuclease whose activation by estrogen correlates with the coordinate destabilization of serum protein mRNAs. This enzyme, named polysomal ribonuclease 1, or PMR-1, is a novel member of the peroxidase gene family. A characteristic feature of PMR-1 is its ability to generate in vitro degradation intermediates by cleaving within overlapping APyrUGA elements in the 5'-coding region of albumin mRNA. The current study sought to determine whether the in vivo destabilization of albumin mRNA following estrogen administration involves the generation of decay intermediates that could be identified as products of PMR-1 cleavage. A sensitive ligation-mediated polymerase chain reaction technique was developed to identify labile decay intermediates, and its validity in identifying PMR-1-generated decay intermediates of albumin mRNA was confirmed by primer extension experiments performed with liver RNA that was isolated from estrogen-treated frogs or digested in vitro with the purified endonuclease. Ligation-mediated polymerase chain reaction was also used to identify decay intermediates from the 3'-end of albumin mRNA, and as a final proof of principle it was employed to identify in vivo decay intermediates of the c-myc coding region instability determinant corresponding to sites of in vitro cleavage by a polysome-associated endonuclease.

The process of mRNA decay is closely linked to translation (1), and a growing body of data links the binding of proteins to the mRNA 3'-end with the efficiency of cap-dependent translation initiation (2, 3). mRNA decay in vertebrates can be initiated by a 22-fold increase in the amount of cellular PMR-1. The purpose of the present study was to determine whether this increase in unit activity is matched by the appearance of albumin mRNA decay intermediates characteristic of endonuclease cleavage by PMR-1.

With the exception of insulin-like growth factor II mRNA, whose unique structure results in a remarkably stable in vivo endonuclease degradation product (10, 16), most mRNAs are degraded without significant accumulation of decay intermediates. However, decay intermediates have been observed using crude in vitro decay systems. The most likely explanation for this is that in vivo these intermediates are subject to rapid exonucleolytic clearance in a manner similar to that which occurs in prokaryotes, where polynucleotide phosphorylase or RNase II rapidly degrade intermediates generated by RNase E (5). For mRNAs that do not show obvious degradation intermediates only a few, highly abundant mRNAs have been examined, in general using primer extension or S1 nuclease protection assays (17). Although these techniques do work, they require long exposure times to visualize metastable products and are not readily applicable to low copy mRNAs. We describe here a new approach using ligation-mediated (LM) RT-PCR to identify the 3'-ends of degradation intermediates generated in vivo as a result of either endonuclease cleavage or pausing of a 3'-to 5'-exonuclease. The rationale behind this was our finding that PMR-1 generates degradation intermediates with 3'-hydroxyls, which are good substrates for further degradation by 3'-to 5'-exonucleases, whereas decay intermediates with 3'-phosphate termini are poor substrates for such enzymes. Using

* This work was supported by Grant GM38277 from NIGMS, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Molecular and Cellular Biochemistry, The Ohio State University, 1645 Neil Ave., Columbus, OH 43210-1218. Tel.: 614-688-3012; Fax: 614-292-7232; E-mail: schoenberg.3@osu.edu.

1 The abbreviations used are: PMR-1, polysomal ribonuclease 1; RT-PCR, reverse transcription-polymerase chain reaction; LM-PCR, ligation-mediated PCR; MEL cells, murine erythroleukemia cells; DTT, dithiothreitol; BSA, bovine serum albumin; nt, nucleotide(s); UTR, untranslated repeat; DEPC, diethylpyrocarbonate; KH, human NK P homology; mRNP, messenger ribonucleoprotein.


This paper is available on line at http://www.jbc.org

12331
Identification of in Vivo mRNA Decay Intermediates

In this assay we show that the estrogen-induced increase in unit activity of polyesosome-bound PMR-1 is accompanied by the appearance of albumin mRNA degradation intermediates corresponding to those generated by in vitro cleavage with the purified endonuclease. As a proof of principle this approach was used to demonstrate in vivo cleavage of c-myc mRNA in the coding region determinant that has been identified from in vitro studies as a potential regulatory element by Ross and coworkers (8, 18).

MATERIALS AND METHODS

Experimental Animals—Male Xenopus One (Ann Arbor, MI), fed a synthetic diet, and maintained in plastic aquaria with a 12-h light-dark cycle. One milligram of estradiol-17β was injected in 0.1 ml of a solution of 10% MeSO/90% propylene glycol into the dorsal lymph sac. Animals were anesthetized with 0.1% tricaine methanesulfonate prior to removing the liver.

Purification of Xenopus Liver RNA—Livers were removed and perfused with ice-cold 1× SSC to remove as much blood as possible. They were chopped into 1-mm cubes followed by the addition of 10 ml/g of tissue of 5 M guanidine isothiocyanate, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM β-mercaptoethanol, and the mixture was homogenized at 4 °C for 2 min at 4000 rpm using a Teflon homogenizer. The homogenate was centrifuged at 12,000 × g for 10 min at 4 °C to remove any insoluble material. One-tenth volume of 20% (v/v) N-lauroylsarcosine was added, and the mixture was heated at 65 °C for 2 min to denature protein. 0.1 g of CaCl2/ml liver extract was added, and the extract was layered over 9 ml of 5 M CsCl in a siliconized polyallomer centrifuge tube and centrifuged overnight at 113,000 × g at 22 °C in a Sorvall TH-641 swinging-bucket rotor. The supernatant was carefully removed, and the RNA pellet was dissolved in 3 ml of 5 mM EDTA, 0.5% (v/v) N-lauroylsarcosine, 5% (v/v) β-mercaptoethanol at 4 °C for 24 h. RNA was extracted once with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with one volume of chloroform:isoamyl alcohol (24:1). Sodium acetate, pH 5.2, was added to 0.3 M and RNA was precipitated with 2.5 volumes of ice-cold ethanol. The recovered RNA pellet was washed with 70% ethanol and dissolved in DEPC-treated water prior to use.

Cell Culture—Murine erythroleukemia cells (MEL cells) were obtained from Dr. Lynne Maquat. They were cultured in Dulbecco’s minimal essential medium containing 10% fetal bovine serum. Total cellular RNA was isolated using TRIzol reagent (Life Technologies, Inc.) following the manufacturer’s protocol.

Primer Ligation—Between 2 and 10 µg of total RNA was added to a 15-µl reaction containing 50 mM Tris, pH 8, 10 mM MgCl2, 20 mM ATP, 2 mM DTT, 10 µg/ml BSA, 1 mM hexamine cobalt chloride, 25% (v/v) polyethylene glycol 8000, 30 units of placental ribonuclease inhibitor, and 2 µg of ligation primer MH11NH3P. The ligation primer MH11NH3P (5'-CCAGGTGGATAGTGCTCAATCTCTAGATCG-NH2) was prepared by Operon and has 5′-phosphate and 3′-amine termini. Ligation reactions were performed for 14,000 × g, 4 °C for 15 min, washed with cold 85% ethanol, and dissolved in 12 µl of distilled H2O.

Four microliters of the extracted DNA was added to a 40-µl reaction containing 4 µl of a 10× buffer consisting of 100 mM Tris-HCl, pH 8.9, 1 mM KCl, 15 mM MgCl2, 500 µg/ml BSA, 0.5% Tween 20 (v/v). The reaction mixture was adjusted to 1.5 mM MgCl2, followed by the addition of 0.4 µl of RNAs, 1 ng of primers MH12, 25 units of RNase digestion buffer III containing 5 units of RNase T1. Samples were incubated for 5–25 min at 37 °C to 39 °C to remove remnant DNA. The reaction was stopped by addition of one-tenth volume of 3 M sodium acetate, pH 5.5, 50 µg of glycogen, and 900 µl of 100% ethanol, followed by chilling at −80 °C. The DNA pellet was recovered by centrifugation at 14,000 × g, 4 °C for 15 min, washed with cold 85% ethanol, and dissolved in 12 µl of distilled H2O.

In vitro Cleavage of the 3′-End of Albumin mRNA—A 5′-32P-labeled transcript corresponding to region G in Fig. 2 (spanning 1690–2002 of albumin mRNA) was prepared as described previously (17). This was incubated with 10 µg of polysomal extract from estrogen-treated frogs (Fig. 5A) or 20 units of PMR-1 purified as described previously (20, 21) (Fig. 5D) in 30 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM MgCl2, and 75 mM KCl. One unit of PMR-1 cleaves 7 fmol of albumin substrate transcript in 30 min at 23 °C. The reactions were stopped by adding one volume of stop solution (98% formamide, 0.1% [v/v] bromphenol blue, and 0.1% xylene cyanol) and heating at 95 °C for 3 min. The samples were then electrophoresed on a 6% polyacrylamide/urea gel and cleavage products were visualized by autoradiography.

RNase T1 Digestion and RNA Structure Modeling—A 5′-32P-labeled transcript corresponding to nt 1690–2002 of albumin mRNA was added to 10 µl of hybridization III buffer from the Ambion RNase Protection Assay III kit (Ambion, Austin, TX). This was heated for 5 min at 50 °C and cooled slowly to room temperature. To this was added 150 µl of RNase digestion buffer III containing 5 units of RNase T1. Samples were incubated for 5–25 min at 37 °C, and the reaction was stopped by addition of 225 µl of RNase inactivation/precipitation III solution. Digested mRNA was recovered by addition of 150 µl of ethanol, and 10 µg of yeast tRNA, followed by precipitation at −20 °C. The pellet was dissolved in 6 µl of gel loading buffer II, and products were separated on a 6% denaturing polyacrylamide/urea gel and visualized by autoradiography. The positions of single-stranded G residues identified by RNase T1 digestion were used to model the secondary structure of this portion of albumin mRNA using the MFOLD server (22).

 Primer Extension— Primer extension analysis of albumin mRNA was performed as described previously (17). 10-µg samples of total liver RNA isolated 12 h after injection of estradiol were either kept on ice or digested as described above with 20 or 40 units of purified PMR-1. The mixtures were heated to inactivate PMR-1, and ethanol was precipitated.
Identification of in Vivo mRNA Decay Intermediates

RESULTS

A Sensitive LM-PCR Assay for Detecting in Vivo mRNA Degradation Intermediates—Earlier work found that degradation intermediates generated by in vitro PMR-1 cleavage of albumin mRNA contained free 3′-hydroxyls, making them susceptible to degradation by 3′- to 5′-exonucleases (23). We took advantage of this observation to develop a generally applicable approach to the identification of in vivo mRNA degradation intermediates. The method diagrammed in Fig. 1 involves ligation of a common primer to the 3′-ends of all RNA molecules followed by reverse transcription primed with a nested complementary primer to generate a population of cDNAs corresponding to all of the primer-tagged RNA fragments in the population. The cDNA is PCR-amplified using a 5′-32P-labeled sense-strand primer specific to the mRNA of interest and the nested primer used for reverse transcription, and the resulting products are separated on a denaturing polyacrylamide/urea gel. Bands identified by autoradiography are excised, re-amplified, and sequenced to identify the 3′-end of the mRNA degradation intermediate at the junction between the ligated primer and the target mRNA. Key to this process is the use of a ligation primer that has been modified by addition of a 5′-phosphate for the ligation to the 3′-hydroxyl termini, and a 3′-amino group to prevent further multimerization of the primer during ligation. This technique does not distinguish between decay intermediates generated by endonuclease cleavage versus those produced by pausing of a 3′- to 5′-exonuclease, but as noted under “Discussion,” the latter have not been detected in mRNAs bearing poly(U) tracts.

LM-PCR Identification of in Vivo Albumin mRNA Decay Intermediates—PMR-1 was identified and purified based on its ability to generate a unique product by cleaving within overlapping APyUGA elements in the 5′-coding region of the molecule (14, 20). Although PMR-1 cleaves preferentially within APyUGA elements, it is not a restriction endonuclease and also cleaves at numerous “nonconsensus” elements (15, 23). Fig. 2 shows a schematic representation of albumin mRNA with the positions of the 14 APyUGA elements indicated in relation to the coding (gray) and noncoding (black) regions. All of these elements fall within the coding region, and there are three pairs of overlapping elements (identified with brackets). Because the in vitro cleavage properties of PMR-1 were characterized with the portion of the 5′-coding region, identified as A in Fig. 2, this sequence was evaluated for the appearance of in vivo degradation intermediates following estrogen administration. Male Xenopus were injected with 1 mg of estradiol, and total liver mRNA isolated 12 or 24 h later was analyzed by LM-PCR using the A1 primer (indicated with a filled horizontal arrow in Fig. 2) beginning at position 40 of albumin mRNA. Lung RNA isolated at time 0 was used as a control. Fig. 3A shows the gel separation of RT-PCR products generated by amplification with 32P-labeled A1 primer. The bands numbered on the right side of the autoradiogram correspond to the cleavage sites mapped in Fig. 3C onto structure of the corresponding region of albumin mRNA. A nonspecific product distinct from albumin mRNA was seen to varying degrees in all samples (filled circle). Two major amplification products from time 0 liver RNA were seen in the lower third of the gel, one of which corresponded to position A14, and the other of which was too close to the sequencing primer to be identified (open circle). Note was the time-dependent appearance of new products 12 and 24 h after estrogen administration (lanes 3 and 4).

Each of the indicated bands was excised from the gel, amplified, and sequenced to identify the junction between the ligated primer and albumin mRNA degradation products. An example of this is shown in Fig. 3B for band A11. The sequence 5′-AUU-primer-3′, corresponded to cleavage 5′-AUU/GAACUGA-3′.
Identification of in Vivo mRNA Decay Intermediates

Fig. 3. LM-PCR identification of decay intermediates in the 5′-coding region of albumin mRNA. A, LM-PCR was performed with 10 μg of total lung RNA (lane 1) or 10 μg of total liver RNA prepared 0, 12, or 24 h after estrogen administration using a 5′-32P-labeled primer complimentary to a portion of the 5′-end of the mRNA. The PCR products were separated on a 6% polyacrylamide/urea gel and visualized by autoradiography. The numbers on the right side of the autoradiogram correspond to the sites shown in C. B, the prominent band A11 was excised, re-amplified, and sequenced to identify the junction between the ligated primer and the mRNA degradation intermediate. C, each of the numbered bands in A was re-amplified and sequenced as in B, and their position is shown on the mapped secondary structure of the 5′-coding region of albumin mRNA bearing the overlapping APyrUGA elements whose in vitro cleavage by PMR-1 was previously characterized (23). The open arrows correspond to in vivo degradation intermediates that were not observed by in vitro cleavage with purified PMR-1.

Previous work characterized PMR-1 as cleaving between the two pyrimidines in the APyrUGA consensus element (23). The absolute identification of cleavage sites afforded by the sequencing of LM-PCR products indicated that this was misplaced by a single nucleotide, a result that likely came from the use of DNA markers and sequencing ladders to size RNA degradation intermediates. The actual cleavage lies between the U and G residues, a result that is consistent with inactivation of cleavage upon mutating APyrUGA to APyrAGA (23). The corrected cleavage sites are indicated in all the figures presented here.

Primer Extension Confirms the LM-PCR Identification of in Vivo PMR-1 Cleavage Sites—In the experiments in Fig. 4 primer extension was used to both validate the LM-PCR identification of in vivo decay intermediates and to show that these resulted from cleavage by PMR-1. In the experiment in Fig. 4A total liver RNA was isolated from control frogs or frogs injected with estradiol 12 h prior to death, and primer extension was performed using a primer for region A indicated by the open arrow above the schematic in Fig. 2. Lanes 3–6 are a DNA sequencing ladder prepared using the same primer and the corresponding albumin cDNA. The positions of the in vivo decay intermediates corresponding to those mapped onto the structure in Fig. 3C are indicated on the left side of the autoradiogram. B, the RNA from 12-h estrogen-treated frogs was incubated for 30 min at 22 °C with (lane 1) or without (lane 2) 40 units of purified PMR-1. One unit of PMR-1 activity equals the amount of enzyme that completely degrades 7 fmol of an albumin mRNA substrate transcript corresponding to region A in 30 min at 22 °C. The positions of the decay intermediates are indicated as in A.
decay intermediates by primer extension (numbered on the side) as those identified in Fig. 3 by LM-PCR.

To determine whether these products resulted from in vivo cleavage by PMR-1 we subjected the 12-h post-estrogen liver RNA to in vitro digestion with purified PMR-1. Because this preparation retained a significant amount of the first 300 nt of albumin mRNA, we reasoned that further in vitro digestion with purified PMR-1 should amplify the signal intensity of products generated by PMR-1 cleavage in vivo. Alternatively, if these decay intermediates were not generated by PMR-1, further in vitro digestion with the purified enzyme would generate a different set of primer extension products. The results of digestion with 40 units of purified PMR-1 are shown in lanes 1 and 2 of Fig. 4B. In vitro cleavage of RNA that was partially “precleaved” in vivo resulted in increased signal intensity for all of the products identified by both LM-PCR and primer extension. Because previous work showed that these decay products are unique to PMR-1 (23) these results indicate that the identified decay intermediates resulted from in vivo cleavage by PMR-1.

Application of LM-PCR to Identify Degradation Intermediates from the 3′-End of Albumin mRNA—Unlike region A in the 5′-coding portion of albumin mRNA, metastable degradation intermediates were never observed in the course of in vitro decay experiments using transcripts from the 3′-end of albumin mRNA (14). Region G (Fig. 2), spanning position 1690 to 2002 of albumin mRNA, contains 173 nt of the coding region and the 3′-310 nt 3′-UTR, and has four APyUGA elements. In vivo cleavage within this sequence was analyzed by LM-PCR in Fig. 5 using the RNA from 24-h estrogen-treated frogs evaluated in Fig. 3. It should be noted that the 6% polyacrylamide gels used here and in Fig. 6 are only capable of resolving cleavage at three of the four APyUGA sites. Eight LM-PCR products were identified in the gel in Fig. 5A, and their positions in the sequence of region G are indicated in Fig. 5B. Site G1 corresponds to the 3′-end of albumin mRNA, and sequencing of this confirmed the presence of the <17-nt poly(A) tail seen in our previous work (24). Like the consensus sites A10 and A11 in the 5′-coding region, there are two overlapping APyUGA elements in this portion of albumin mRNA (sites G2 and G3). However, neither of the LM-PCR products corresponding to cleavage at G2 and G3 nor the product for cleavage at the other APyUGA element G7, were as prominent as that seen for A11 in Fig. 3A.

In Vitro Cleavage of the 3′-End of Albumin mRNA—To determine why results with region G did not match those seen with region A, we determined the pattern of in vitro cleavage of region G of albumin mRNA using 5′-end-labeled transcript in a manner similar to that used earlier to study cleavage within region A (23). In the experiment in Fig. 6A, region G transcript was incubated with polysome extract from estrogen-treated frogs prepared as described previously (14). Degradation intermediates corresponding to the in vivo cleavage sites identified in Fig. 5 are labeled on the right. Although numerous bands were seen that are typical for in vitro assays, the main pattern was similar to that observed for in vivo decay by LM-PCR. This experiment was repeated in Fig. 6B using 20 units of purified PMR-1 instead of polysome extract. Here only two major cleavages were observed, one at site G7, which contains a consensus APyUGA element, and one at the adjacent site G8. Based on results obtained in Figs. 3 and 4, we suspect that 1) the better correspondence between the cleavage pattern seen with polysome extract and in vivo decay intermediates either resulted from the presence of auxiliary proteins in the extract that potentiate the ability of PMR-1 to cleave this portion of albumin mRNA or 2) some of these bands are products of additional steps in mRNA decay.
Identification of in Vivo mRNA Decay Intermediates

Impact of Secondary Structure on PMR-1 Cleavage of the Albumin mRNA 3' End—When APyrUGA is present in a single-stranded conformation, it is the predominant site for PMR-1 cleavage in both albumin (14) and vitellogenin mRNA (25); however, PMR-1 is unable to cleave within the element when present in double-stranded RNA (23). To test whether the lack of strong cleavages within APyrUGA sites in region G was due to RNA secondary structure, a 5'-end-labeled transcript was digested with limiting amounts of RNase T1 and the identified single-stranded G residues were used to model this sequence to secondary structure using MFOLD. The RNase T1 cleavage sites appear as H in the structure, and the eight cleavage sites mapped in vivo and in vitro are labeled by the arrows. The four APyUGA sequences present are identified by the boxes. Note that the APyrUGA site in the bottom left portion of the structure was not resolved in the gels used in Figs. 5–7.

FIG. 7. Secondary structure of the 3'-end of albumin mRNA. The 5'-32P-labeled transcript for the albumin mRNA 3'-end was incubated with 5 units of RNase T1, and the products were separated on a denaturing 6% polyacrylamide/urea gel. Lanes 1 and 2 correspond to 10 and 15 min of digestion. The positions of cleaved G residues (identified by dots on the autoradiogram) were determined by mobility relative to size standards. These were used to guide the generation of a predicted secondary structure using MFOLD. The RNase T1 cleavage sites appear as H in the structure, and the eight cleavage sites mapped in vivo and in vitro are labeled by the arrows. The four APyUGA sequences present are identified by the boxes. Note that the APyrUGA site in the bottom left portion of the structure was not resolved in the gels used in Figs. 5–7.

Ability of the Coding Region Determinant to Influence mRNA Stability—In addition, these results demonstrate that the LM-PCR approach to mapping in vivo mRNA decay intermediates is generally applicable to both highly abundant mRNAs like albumin, and rare mRNAs like c-myc.

FIG. 8. LM-PCR mapping of in vitro degradation intermediates within the c-myc coding region determinant. A, LM-PCR was performed as in Fig. 3 with 2 μg of total RNA isolated for MEL cells using a primer specific to a region upstream of the c-myc CRD. The PCR products were separated on a 6% acrylamide/urea gel, and the bands of interest, shown by the arrows, were excised out and sequenced. B, the region of c-myc mRNA containing the CRD is shown with the arrows indicating the locations of the in vivo degradation intermediates identified in A. The underlined region corresponds to the region previously shown to be cleaved in vitro by a polysome-associated endonuclease (8).

Identification of Decay Intermediates Consistent with in Vivo Endonuclease Cleavage within the c-myc CRD—Ross and co-workers identified an mRNA instability determinant within the coding region of c-myc mRNA (26) that is both a site for in vitro cleavage by a polysome-associated endonuclease activity (8) and for binding by a KH-domain protein (18). Because little was known about the relationship between the CRD and the degradation of c-myc in vivo, we chose to examine cleavage within the CRD as a test of the ability of LM-PCR to detect labile in vivo decay intermediates from a rare and inherently unstable mRNA. In the experiment in Fig. 8 LM-PCR was performed on 2 μg of total RNA isolated from murine erythroleukemia (MEL) cells using a gene-specific primer complementary to nucleotides 1669–1687 of c-myc mRNA. A denaturing polyacrylamide gel of the 32P-labeled LM-PCR products is shown in Fig. 8A, and the sequence of this portion of c-myc mRNA showing the locations of the five identified in vivo mRNA degradation intermediates is shown in Fig. 8B. As with albumin mRNA, each of these sites was determined by the sequence of the junction between the ligated primer and the c-myc mRNA degradation intermediate. Band 4 is particularly noteworthy here, because this corresponds to a previously mapped site for in vitro cleavage of c-myc mRNA (8), thus supporting the notion that the in vivo degradation of c-myc mRNA involves endonucleolytic cleavage. In addition, these results demonstrate that the LM-PCR approach to mapping in vivo mRNA decay intermediates is generally applicable to both highly abundant mRNAs like albumin, and rare mRNAs like c-myc.

DISCUSSION

With few exceptions, mRNA degradation in vivo is not accompanied by the appearance of stable degradation intermediates. The most likely explanation for this is that in vivo these intermediates are subject to rapid exonucleolytic clearance. However, decay intermediates are observed using crude in vitro decay systems, and the similarity seen between in vivo
and in vitro decay intermediates from the 3'-end of albumin mRNA in Figs. 5 and 6 underscores the usefulness of such systems in recapitulating steps in mRNA decay. Primer extension and S1 protection assays have been used successfully to identify in vivo endonuclease cleavage of apo-very low density lipoprotein II mRNA (7) and transferrin receptor mRNA (9), and we have used these approaches to demonstrate in vivo cleavage within the APyrUGA consensus PMR-1 cleavage sites in region A of albumin mRNA both here and in a previous report (17). However, these techniques work best with highly abundant mRNAs, and even with these its long exposure times may be necessary to visualize some decay intermediates. In this report we introduce ligation-mediated PCR for the rapid and precise mapping of in vivo mRNA decay intermediates. The advantages offered by LM-PCR include its ease of implementation, scalability, and ability to identify degradation intermediates from even rare mRNAs such as c-myc. Although LM-PCR and primer extension were used in this study to confirm the involvement of PMR-1 in the in vivo endonucleolytic degradation of albumin mRNA, by definition, LM-PCR cannot distinguish between the 3'-end of a decay product generated by endonuclease cleavage versus a pausing site for a 3'-5'-exonuclease. That being said, Shyu and coworkers (Department of Biochemistry and Molecular Biology, University of Texas Medical Center, Houston, TX) were unable to demonstrate in vivo exonuclease pausing at poly(G) tracts as has been observed by Parker and coworkers for yeast mRNA decay (27). This leads us to conclude that the decay intermediates identified here, particularly for c-myc mRNA, are the products of endonuclease cleavage.

Previous work from our laboratory identified PMR-1 as an estrogen-induced endonuclease whose activity appeared on polysomes coincident with the estrogen-induced destabilization of albumin and other serum protein mRNAs (14). Using antibodies to PMR-1 we recently found that this mRNA endonuclease resides on polysomes in a latent form that can be released with EDTA as part of a >670-kDa mRNP complex.2 Estrogen induces a 22-fold increase in unit activity of polysome-bound PMR-1, which is accompanied by the coordinate disappearance of both albumin mRNA and PMR-1 from polysomes. The identification of estrogen-induced in vivo decay intermediates in Figs. 3 and 4 corresponding to products of PMR-1 cleavage lends considerable support to a central role for PMR-1 in catalyzing the destabilization of albumin mRNA.

We recently demonstrated that vitellogenin mRNA, which is induced and stabilized by estrogen, contains two APyrUGA elements in the 3'-UTR that are cleaved by PMR-1. The vitellogenin mRNA 3'-UTR is bound by vigilin, a 155-kDa estrogen-induced multi-KH domain protein (28), and this binding inhibits its in vitro cleavage by PMR-1 (25). High affinity binding by vigilin requires a relatively unstructured target sequence, and although albumin mRNA region A contains the same APyrUGA PMR-1 cleavage sites as the vitellogenin mRNA 3'-UTR, vigilin binds poorly to this highly structured sequence and is unable to protect it from PMR-1 cleavage. Results presented here show significantly greater in vitro and in vivo cleavage within the paired APyrUGA sites in the single-stranded loop of albumin mRNA region A than with the same paired element in a hairpin structure in region G. Together these data point to the interplay between primary sequence and secondary structure of an mRNA target both for determining susceptibility to cleavage by an mRNA endonuclease and for mRNA stabilization resulting from protein binding to these sites.

Finally, we examined the degradation of c-myc mRNA as a proof of principle for the applicability of the LM-PCR approach to the identification of in vivo mRNA decay intermediates. The experiment in Fig. 8 focused on the coding region determinant in c-myc mRNA that has been extensively characterized in vitro by Ros and coworkers as a site for ribosome pausing, endonuclease cleavage, and protection from cleavage by the binding of a KH-domain protein (8, 18, 29, 30), but whose cleavage in vivo had yet to be demonstrated. Our results identified degradation intermediates in this region, one of which (site 4) corresponds to the major site for in vitro cleavage by a polysome-associated endonuclease (8). These results lend further validity to the use of in vitro systems for analyzing the biochemistry of mRNA decay and underscore the general applicability of LM-PCR to the identification of in vivo decay intermediates.

Acknowledgment—We thank Elena Chernokalskaya for her help with the primer extension experiments in this study.

REFERENCES

A.-B. Shyu, personal communication.
Identification of in Vivo mRNA Decay Intermediates Corresponding to Sites of in Vitro Cleavage by Polysomal Ribonuclease 1
Mark N. Hanson and Daniel R. Schoenberg


Access the most updated version of this article at doi: 10.1074/jbc.M010483200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 27 references, 13 of which can be accessed free at http://www.jbc.org/content/276/15/12331.full.html#ref-list-1