

Characterization and Purification of a Mammalian Endoribonuclease Specific for the α -Globin mRNA*

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The α -globin mRNA has previously been shown to be the target of an erythroid-enriched endoribonuclease (ErEN) activity which cleaves the mRNA within the 3'-untranslated region. We have currently undertaken a biochemical approach to purify this enzyme and have begun characterization of the enzyme to determine requirements for substrate recognition as well as optimal cleavage conditions. Through mutational analysis and truncations we show that a 26-nucleotide region of the α -globin 3'-untranslated region is an autonomous element that is both necessary and sufficient for cleavage by ErEN. Mutations throughout this region abolish cleavage activity by ErEN suggesting that the entire sequence is important for recognition and cleavage. ErEN is most active under biological salt concentrations and temperature and activity of the enzyme does not require cations. The size for ErEN was estimated by denaturing gel filtration analysis and is ~40 kDa. Interestingly, the exquisite specificity of ErEN cleavage became compromised with increased purity of the enzyme suggesting the involvement of other proteins in specificity of ErEN cleavage. Nondenaturing gel filtration of MEL extract demonstrated that ErEN is a component of an ~160 kDa complex implying that additional proteins may regulate ErEN activity and provide increased cleavage specificity.

In recent years it has become increasingly clear that post-transcriptional regulation plays an important role in gene expression. It is also clear that all levels of mRNA production are highly regulated, including mRNA turnover. A number of elements contribute to the stability of a given mRNA and these include the m⁷G cap at the 5' termini of the mRNA and the poly(A) tail at the 3' termini of the transcript (1, 2) which provide a basal level of stability. Specific *cis* elements, in addition to these general factors, also contribute to mRNA turnover rates. Stability elements of many mRNAs are often located within the 3'-untranslated region (3'-UTR)¹ of the transcript, including the stable globin genes, α -globin and β -globin, as well as very short-lived transcripts such as c-Myc (3–7).

The pathways responsible for mRNA decay have been pri-

marily worked out in the yeast *Saccharomyces cerevisiae*. In yeast, the predominant mechanism of mRNA decay involves deadenylation followed by decapping by DCP1p and subsequent 5'-3' exoribonuclease decay of the transcript by the exoribonuclease XRN1p (8–13). Another pathway in which the transcript is deadenylated and then digested in the 3'-5' direction has also been identified (14, 15).

Recently, due mainly to the development of reliable *in vitro* decay assay systems utilizing mammalian cell extracts, the pathways of mRNA turnover in higher eukaryotes are becoming clearer. A poly(A)-specific 3'-5' exoribonuclease has recently been cloned and encodes a protein termed poly(A) ribonuclease (16). Poly(A) ribonuclease 3'-5' exoribonuclease has been shown to be the major deadenylase responsible for deadenylation in some *in vitro* decay systems (17). A mammalian homolog of XRN1 has been identified which preferentially digests G4 tetraplex substrates, however, its role in mRNA turnover is unclear (18). A complex of 3'-5' exoribonucleases, termed the exosome, were recently discovered in yeast and appear to be involved mainly in ribosomal RNA processing, however, a subset have been shown to be important in mRNA turnover (14, 15, 19). Homologs for 9 of the 11 yeast exosome proteins have been identified in mammals and two of them correspond to autoantigens for antibodies produced by patients with polymyositis-scleroderma overlap syndrome (20, 21). Two distinct decapping activities have also been identified in mammalian cell extracts including a putative DCP1-like activity (22) as well as a second decapping activity that functions as a scavenger which hydrolyzes the residual cap structure following exoribonucleolytic decay of the mRNA (23, 24,46). It appears that following deadenylation, the major decay pathway in mammalian cells proceeds through 3' to 5' exoribonucleolytic degradation while, degradation from the 5' end is a minor contributor (24).

Endoribonucleases have also been identified to play a role in the turnover of specific mRNA transcripts. To date, endoribonucleolytic cleavage intermediates have been described for RNAs including the transferrin receptor, 9E3, IGF-II, ApoII, *Xenopus* Xlhbox2B mRNA, albumin, c-Myc, vitellogenin, hepatitis B virus, and α -globin (25–34, 46). Of these endoribonucleases, two have been cloned which are GAP-SH3-binding protein that cleaves c-Myc and PMR-1, the endoribonuclease that cleaves *Xenopus* albumin mRNA as well as the vitellogenin mRNA (34–36).

The globin mRNAs are among the more stable transcripts and much has been learned about components involved in their stability. The α -globin mRNA has been shown to contain a C-rich region in its 3'-UTR which confers stability on the transcript (37, 38). A ribonucleoprotein complex which binds to this C-rich region, termed the α -complex, has been shown to stabilize the α -globin 3'-UTR (α 3'-UTR) through an interaction with the poly(A)-binding protein (PABP) that is bound to the

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¹ The abbreviations used are: 3'-UTR, 3'-untranslated region; ErEN, erythroid-enriched endoribonuclease; MEL, murine erythroleukemia; α 3'-UTR, α -globin 3'-untranslated region; nt, nucleotide; MERCs, minimal ErEN recognition and cleavage sequence; β 3'-UTR, β -globin 3'-untranslated region.

poly(A)-tail of the transcript (37, 39). It has also been demonstrated that the α -complex protects the $\alpha 3'$ -UTR from cleavage by an erythroid enriched endoribonuclease termed ErEN (30). Protection of an mRNA from endoribonuclease cleavage by trans factors is likely to emerge as a common theme. The vitellogenin mRNA has recently been shown to be protected from endoribonucleolytic cleavage by PMR-1 when bound by the RNA-binding protein vigilin (34). Similarly, specific protein factors also appear to bind and shield the transferrin receptor, c-Myc, and Xlhbox2A mRNAs from endoribonuclease attack (26–28).

There are several aspects about ErEN activity that are unusual when compared with other endoribonucleases. First is its dependence on the poly(A)-tail. ErEN cleavage of the $\alpha 3'$ -UTR requires deadenylation of the RNA prior to endoribonuclease cleavage (37). Deadenylation is essential to disrupt the α -complex-poly(A)-binding protein interaction and reduce the affinity of the α -complex for the $\alpha 3'$ -UTR thus allowing ErEN to displace the α -complex and cleave the RNA (37). For other mRNAs that have thus far been characterized, deadenylation does not seem to be a prerequisite for endoribonucleolytic cleavage (26, 27, 29). Second, the cleavage of the $\alpha 3'$ -UTR by ErEN appears to be a sequence-specific cleavage and has been mapped to nucleotide 63 within the $\alpha 3'$ -UTR (30). A mutation of 6 nucleotides (nt) surrounding the $\alpha 3'$ -UTR cleavage site completely blocks targeting of ErEN to the $\alpha 3'$ -UTR (30). Among the other vertebrate endoribonucleases characterized, only one other has been shown to be sequence-specific when multiple binding sites were reiterated into a heterologous RNA and that is the 120-kDa protein which cleaves the Xlhbox2 mRNA (27). However, the gene encoding this protein remains to be identified. GAP-SH3-binding protein cleaves the c-Myc 3'-UTR at CA dinucleotides and PMR-1, which cleaves the *Xenopus* albumin mRNA, has been shown to be sequence selective, in that it preferentially cleaves at AYUGA sequences but is not sequence-specific since it also cleaves at other single stranded regions (31, 40). We have initiated the purification of ErEN, have identified the minimal cleavage site requirements, and characterized the conditions of ErEN cleavage.

EXPERIMENTAL PROCEDURES

Extract Preparation—Murine erythroleukemia (MEL) and HeLa cells were purchased from the National Cell Culture Center (Minneapolis, MN). Cell pellets were resuspended in 1.5 ml/10⁸ cells of buffer A (10 mM Tris, pH 7.5, 1 mM KOAc, 1.5 mM MgOAc, 2 mM dithiothreitol). Cells were lysed with 25 strokes of a type-B Dounce homogenizer and nuclei were pelleted with a 15-min 4,000 $\times g$ centrifugation. The supernatant was layered over buffer A containing 30% (w/v) sucrose and centrifuged at 130,000 $\times g$ for 1[1,2] h. The resulting supernatant (S130) was removed without disturbing the S130-sucrose interface, supplemented with glycerol to a final concentration of 10% (v/v), and frozen at -70°C in aliquots.

RNA Production—The template for the α -globin 3'-UTR was PCR amplified from the pSV2Aneo- $\alpha 2$ plasmid as previously described (41) with primers that introduce a T7 bacteriophage promoter at the 5' end. Templates for the $\alpha 3'$ -UTR *Hind*III mutants were PCR amplified using the corresponding mutant template plasmid and the same set of primers used for the wild type 3'-UTR. The H13, H15, H17, H19, H21, and H23 PCR templates were amplified from the plasmids pSV2A- α H13, α H15, α H17, α H19, α H21, and α H23, respectively (kindly provided by Dr. S. Liebhaber; University of Pennsylvania). These plasmids have been previously described (38). Truncations of the $\alpha 3'$ -UTR were PCR amplified from pSV2Aneo- $\alpha 2$ using 5' primers that incorporated a T7 bacteriophage promoter and are listed as follows: 37–81 (CGTAATACGACTCACTATAGGGCCTCCCAACGGGC); 44–81 (CGTAATACGACTCACTATAGGGAACGGGCCCTCC); 50–81 (CGTAATACGACTCACTATAGGGCCCTCCCTCCC); 56–81 (CGTAATACGACTCACTATAGGGTCCCTCCCTG) and the following 3' primer (CAGGAAGGCCGGTG). The GA mutant templates were PCR amplified using extended 3' primers which replaced the wild type sequence with GAGAGA at the indicated positions. The PCR products were

phenol/chloroform extracted twice and chloroform extracted twice, ethanol precipitated, and washed with 70% ethanol. RNA transcripts were generated using T7 polymerase (Promega) according to the manufacturer's conditions using 500 ng of template. The full-length $\alpha 3'$ -UTR and *Hind*III RNAs were uniformly labeled by including [α -³²P]UTP in the reaction and the truncated transcripts and GA mutants were labeled by including [α -³²P]CTP in the reaction. All RNAs were gel purified as described in Wang *et al.* (41).

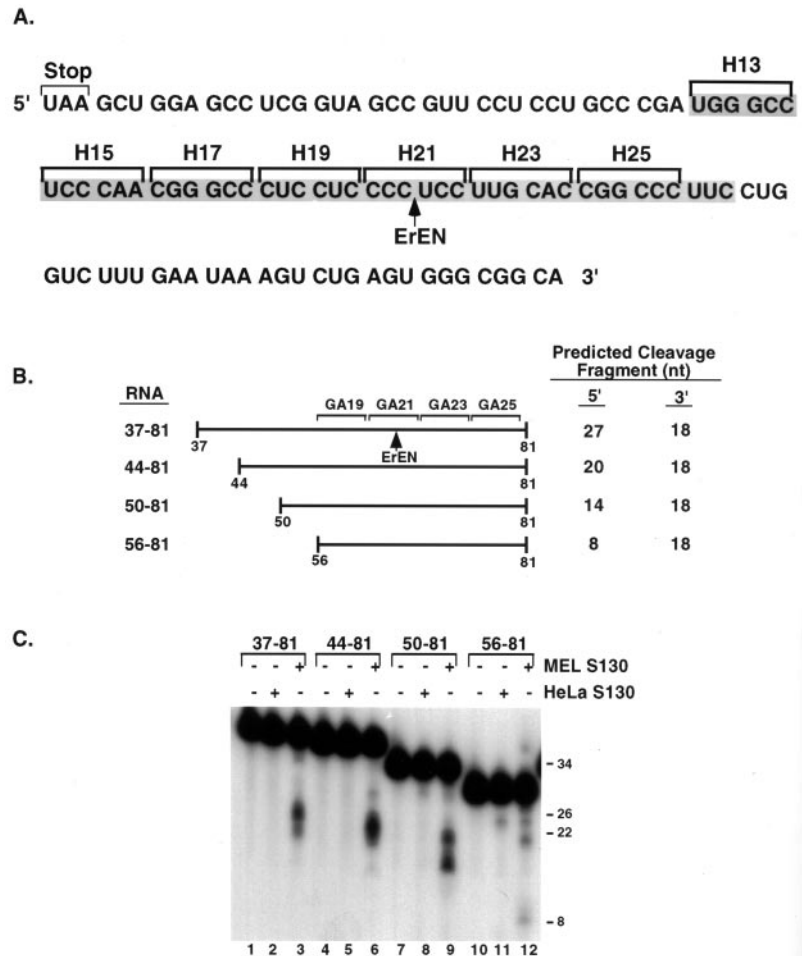
To generate 5' end-labeled RNA, unlabeled RNA without a 5' cap was made and vaccinia virus capping enzyme was used to label the 5' end with [α -³²P]GTP as previously described (41). An alkaline RNA ladder was prepared by incubation of 10⁴ cpm of 5' end-labeled RNA in 50 mM NaOH and 10 mM EDTA for 20 s at 95 $^\circ\text{C}$. Reactions were stopped by the addition of an equal volume of 9.5 M urea, 80 mM NaOAc, and 170 mM HOAc.

In Vitro mRNA Decay Assays—Decay reactions were carried out as previously described (41) using 0.1 pmol of ³²P uniformly labeled RNA. The RNA was incubated with 20 μg of the indicated protein for 20 min at room temperature (unless otherwise noted) in IVDA buffer (10 mM Tris, pH 7.5, 100 mM KOAc, 2 mM MgOAc, 5 mM EDTA, 2 mM dithiothreitol, 10 mM creatine phosphate, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine) containing 8 units of RNasin. In experiments to test optimal salt concentrations IVDA buffer without KOAc was used and KOAc was added to the final concentrations indicated. In experiments to test cation requirement, EDTA was omitted as indicated, IVDA buffer lacking MgOAc was used and cations were added as labeled. To determine the nature of the cleavage product, RNA that had been cleaved with 20 μg of protein from the octyl-Sepharose fraction and phenol/chloroform extracted was incubated with 50 ng of either the Int II/FL primer (5'-TTTTTTTTTTTTTTTTTTTTTTTTTTGGCCCTCCAGACTTT-3') or the Int I primer (5'-GAGGAGGGGAGGAACATGTTTCGAACG-3') with 40 units of RNasin (Promega) in 10 mM Tris, pH 7.5, 5 mM MgCl₂, and 7.5 mM dithiothreitol at 65 $^\circ\text{C}$ for 10 min, cooled slowly to room temperature to allow annealing of the primer to the RNA and then incubated for 15 min at 25 $^\circ\text{C}$ with 0.25 mM dNTPs and 0.2 unit of Klenow (New England BioLabs).

Biochemical Fractionation—Gel filtration under denaturing conditions was carried out using ~ 750 μg of MEL S130 extract adjusted to 2 M urea. The denatured protein was loaded onto a HiPrep 16/20 Sephacryl S-200 column (Pharmacia Corp.) that had been equilibrated in HG (20 mM HEPES, pH 7.5, and 10% glycerol) with 40 mM KCl (HG-40) containing 2 M urea. For all column purifications an LCC-500 HPLC was used (Pharmacia Corp.). Half-milliliter fractions were collected and half of every third fraction was dialyzed and concentrated against HG-40 in microcon YM-10 centrifugal concentrators (Millipore) prior to use in decay assays. Gel filtration under native conditions was carried out using ~ 750 μg of MEL S130 extract that was adjusted to 1 mM CaCl₂ and incubated with 200 units of micrococcal nuclease (Pharmacia Cor.) per ml of extract at 30 $^\circ\text{C}$ for 15 min. The reactions were stopped with the addition of 5 mM EGTA and spun at 4 $^\circ\text{C}$ for 5 min. The resulting supernatant was loaded onto a Superose 12 HR 10/30 column (Pharmacia Corp.) that had been equilibrated in HG-40 and 0.5-ml fractions were collected. Half of every second fraction was concentrated as described above and used directly in decay assays. Separately, gel filtration standards (Bio-Rad) were loaded onto the above columns under the same conditions to determine the correlation between molecular weight and fraction number.

MEL S130 extract was isolated as described above. Extract was adjusted to 1 M (NH₄)₂SO₄ and precipitated protein was separated from the supernatant by centrifugation at 4,000 $\times g$ for 20 min. The resulting supernatant was loaded directly onto a HiPrep octyl-Sepharose column (Pharmacia Corp.) that had been equilibrated in 1 M (NH₄)₂SO₄, 50 mM Na₂HPO₄, pH 7.5. Proteins were eluted in steps with 0.6 M (NH₄)₂SO₄, 50 mM Na₂HPO₄, pH 7.5, buffer and then 50 mM Na₂HPO₄, pH 7.5, buffer. The 0.6 M (NH₄)₂SO₄ fraction was adjusted to 1.7 M (NH₄)₂SO₄ and precipitated proteins were removed by centrifugation as above. The supernatant was dialyzed to HG-40 and loaded onto an SP-Sepharose column (Pharmacia Corp.). Proteins were eluted in steps at HG-200 and HG-600. The 0.6 M KCl fraction was dialyzed against HG-40 and loaded onto a HiPrep Heparin column (Pharmacia Corp.), washed with HG-200 followed by a 15-ml gradient from 0.2 to 0.7 M KCl. One-milliliter fractions were collected, dialyzed against HG-40, and assayed for ErEN activity after which the fractions containing activity were pooled and adjusted to 50 mM KCl and 10 mM Na₂HPO₄. The resulting protein was loaded onto a hydroxyapatite column (Bio-Rad) that had been equilibrated in HAB (20 mM HEPES, pH 7.5, 50 mM KCl, and 10% glycerol) with 10 mM Na₂HPO₄. Bound protein was eluted with a 15-ml gradient from 10 to 300 mM Na₂HPO₄. Fractions (0.5 ml) were collected, dialyzed

FIG. 1. Identification of a 26-nt ErEN recognition sequence. A, the sequence of the 110-nt $\alpha 3'$ -UTR is shown. The ErEN cleavage site, at nucleotide 63, is designated with an *arrow* and the *shaded region* corresponds to a previously identified 45-nt segment that was able to target a heterologous RNA for ErEN cleavage. The positions of previously described *Hind*III substitution mutants (38) are indicated by the *brackets* and the designation of the mutant is listed *above* each bracket. B, constructs used to identify the 26-nt region of the $\alpha 3'$ -UTR required for ErEN cleavage are shown. The 5' and 3' ends are numbered *below* each construct according to their position within the $\alpha 3'$ -UTR. The region of the $\alpha 3'$ -UTR included in each RNA is listed on the *left* and the predicted sizes of the 5' and 3' fragments resulting from ErEN cleavage are listed on the *right* in nucleotides. The ErEN cleavage site is designated with an *arrow* and the *brackets above* indicate positions of GAGAGA substitution mutants listed with the same nomenclature as in A above. C, an *in vitro* decay assay was carried out using uniformly labeled RNA described in B. Inclusion of either HeLa S130 or MEL S130 extract is indicated. The first lane of each set shows the migration of the input RNA. The *numbers above the brackets* represent the RNA fragments listed in *panel B* and the position of the DNA size markers are indicated on the *right* in nucleotides.



against HG-40, and a portion was used in activity assays as well as separated by SDS-PAGE and the protein profile visualized by silver staining.

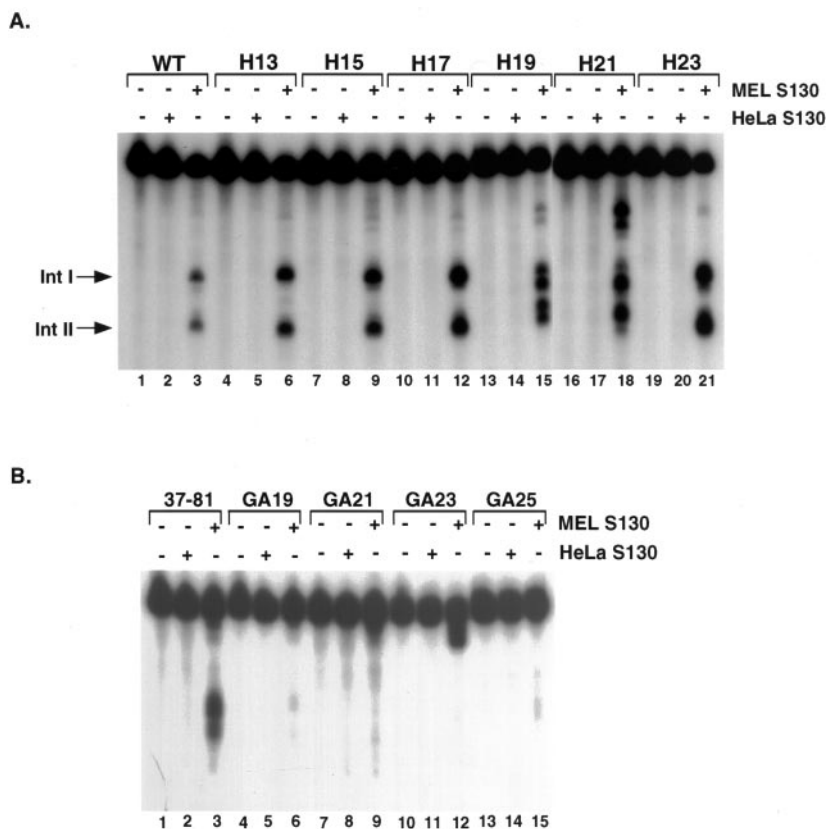
RESULTS

26 Nucleotides of the $\alpha 3'$ -UTR Are Required for ErEN Recognition and Cleavage—We have identified a sequence-specific erythroid-enriched endoribonuclease (ErEN) which specifically targets and regulates the α -globin mRNA. The cleavage site within the $\alpha 3'$ -UTR has previously been mapped to nucleotide 63 within the 110-nt $\alpha 3'$ -UTR (Fig. 1A) (30). This site falls within the region of the $\alpha 3'$ -UTR that is bound by the α -complex (42). Binding of the α -complex to this region prevents access of ErEN to the cleavage site and results in stabilization of the RNA (37). Furthermore, a 45-nt region spanning nucleotides 37–81 (*shaded* in Fig. 1A) was shown to be an autonomous element which, when placed onto a heterologous RNA, could still be recognized and specifically cleaved by ErEN (30). In an effort to identify the minimal ErEN recognition and cleavage sequence (MERCS) we first tested whether ErEN is able to cleave a 45-nt RNA from nucleotides 37 to 81 of the $\alpha 3'$ -UTR directly without it being in the context of a longer RNA. The expected 5' fragment of the ErEN cleavage product would be 27 nt and the expected 3' fragment would be 18 nt (Fig. 1B). Since HeLa cells do not contain ErEN activity (30), HeLa S130 extract was used as a negative control to identify any background nuclease activity. As expected, no cleavage was detected with HeLa S130 extract (Fig. 1C, *lane 2*), however, two fragments of the expected sizes were detected with MEL S130 extract (*lane 3*). A series of truncations which removed sequences from the 5' end of this 45-nt region were generated (Fig. 1B) and each tested in a decay assay (Fig. 1C).

All of the constructs gave the expected cleavage fragments. Surprisingly, the smallest probe tested, which encompasses 26 nt, was also recognized and cleaved by ErEN albeit at a reduced efficiency (Fig. 1C, *lane 12*). Addition of random sequence to the 5' end of the 26-nt RNA, enhanced cleavage of this longer RNA (data not shown) suggesting that there is a minimal size constraint for ErEN.

To determine the essential sequences for ErEN cleavage, we tested the ability of ErEN to cleave mutant $\alpha 3'$ -UTR sequences. A series of scanning substitution mutants that change 6 nt at a time to a *Hind*III (38) throughout the $\alpha 3'$ -UTR were initially tested. The positions of the mutations are shown in Fig. 1A and the nomenclature is that used by Weiss and Liebhaber (38). The numbers represent sets of triplicate nucleotides that have been substituted by a *Hind*III recognition sequence within the $\alpha 3'$ -UTR following the stop codon. For example, H13 substitutes nucleotides 37–42 of the $\alpha 3'$ -UTR which would correlate to the 13th and 14th codon in the $\alpha 3'$ -UTR if it were translated. Uniformly labeled $\alpha 3'$ -UTR RNAs were made from each construct and tested in decay assays with MEL S130 cell extract. No products are detected using HeLa S130 extract with any of the $\alpha 3'$ -UTR substrates (Fig. 2A, *lanes 2, 5, 8, 11, 14, 17, and 20*). Four of the mutations have no detectable effect on ErEN cleavage and only the H19 and H21 mutants, which span nucleotides 55–66, alter ErEN cleavage of the $\alpha 3'$ -UTR (*lanes 15 and 18*). Consistent with the deletional analysis in Fig. 1, these two mutations fall within the above identified 26-nt MERCS. Surprisingly, cleavage is not completely abolished by these mutants but rather the specificity of the cleavage appears to be altered. A possible explanation is that since the *Hind*III mutation retains a CUU in this CU-rich region, ErEN recogni-

FIG. 2. Sequence requirements for ErEN cleavage. A, uniformly labeled $\alpha 3'$ -UTR and mutant $\alpha 3'$ -UTR RNAs containing hexanucleotide substitutions that introduced a *Hind*III site were used in decay assays with either HeLa S130 or MEL S130 extract as labeled. The RNA used in each set is designated above the brackets. The positions of the wild type 5' and 3' cleavage intermediates are labeled on the left as Int I and Int II, respectively. B, *in vitro* decay assays were carried out using either MEL or HeLa extracts as denoted and uniformly labeled RNAs as indicated above the brackets. All RNAs are from nucleotides 37 to 81 and those labeled GA have a 6-nt substitution of GAGAGA. GA19 substitutes nucleotides 55 to 60, GA21 substitutes nucleotides 61 to 66, GA23 substitutes nucleotides 67 to 72, and GA25 substitutes nucleotides 73 to 78.



tion and cleavage of the $\alpha 3'$ -UTR is only modestly altered.

To test whether more severe mutations could disrupt ErEN cleavage activity, a second set of substitution mutants were generated in which a series of hexanucleotide substitution mutations within the 26-nt MERCS region were made. The mutants were generated within the context of the 45-nt RNA to ensure the detection of sufficient cleavage activity. The substitutions introduced the sequence GAGAGA. This sequence was chosen because it was previously shown to disrupt ErEN cleavage activity when positioned at the cleavage site within the context of the full-length α -globin 3'-UTR (30). As shown in Fig. 2B, introduction of any of these mutations completely abolished ErEN cleavage by MEL S130 extract (Fig. 2B, lanes 6, 9, 12, and 15). These data suggest that the entire 26-nt MERCS region is necessary and sufficient for cleavage by ErEN in MEL S130 extract.

Partial Purification of ErEN Activity from MEL Cells—In an effort to characterize ErEN, we determined the approximate size of the protein containing ErEN activity. Gel filtration was carried out with MEL S130 extract under denaturing conditions with 2 M urea. The proteins were separated on a Sephacryl S-200 column which allows the separation of proteins from 5 to 250 kDa. Fractions from the column were dialyzed to remove the urea and renature the proteins. Decay assays were then carried out on the individual fractions using the $\alpha 3'$ -UTR as substrate (Fig. 3). Protein standards were also separated by gel filtration in the presence of 2 M urea and compared with the ErEN fractions to assess the approximate size of the protein. As seen in Fig. 3, ErEN activity peaks in the fractions that correspond to ~40 kDa (lanes 8 and 9) indicating that ErEN is likely to be a single protein in that size range.

We undertook a biochemical approach to purify ErEN activity from cells. The starting material was MEL cells in which ErEN activity is readily detectable. The purification scheme is outlined in Fig. 4A and proteins from each of the fractions containing ErEN activity are shown on a silver-stained gel in

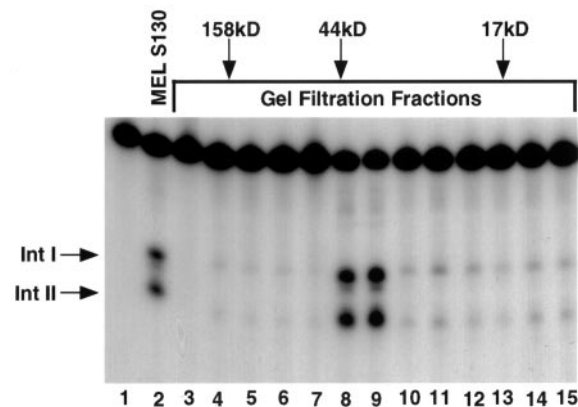


FIG. 3. ErEN activity corresponds to a protein ~40 kDa in size. Gel filtration was carried out with 2 M urea using 750 μ g of MEL S130 extract and the fractions were assayed for ErEN activity (lanes 3–15). The activity of renatured MEL S130 extract is shown in lane 2 and the cleavage products are labeled as described in the legend to Fig. 2. The migration of gel filtration protein standards are shown with arrows above the bracket.

Fig. 4B along with the activity assay from each fraction. The first step in the purification was the isolation of S130 extract from MEL cells which removes nuclei and polysomes since they appear to be deficient in ErEN activity (30). The extract was subsequently adjusted to 1 M $(\text{NH}_4)_2\text{SO}_4$ and the supernatant was bound to an octyl-Sepharose column. Proteins were eluted in steps by decreasing the $(\text{NH}_4)_2\text{SO}_4$ concentration. ErEN activity was eluted with 0.6 M $(\text{NH}_4)_2\text{SO}_4$ (lane 4), the concentration of $(\text{NH}_4)_2\text{SO}_4$ was adjusted to 1.7 M, and the supernatant which contains ErEN activity was collected (lane 5). Following dialysis, to remove the $(\text{NH}_4)_2\text{SO}_4$, the proteins were bound to SP-Sepharose and batch eluted with 0.6 M KCl (lane 6). The eluate was desalted and bound to heparin-Sepharose. The column was washed with 0.2 M KCl and proteins eluted

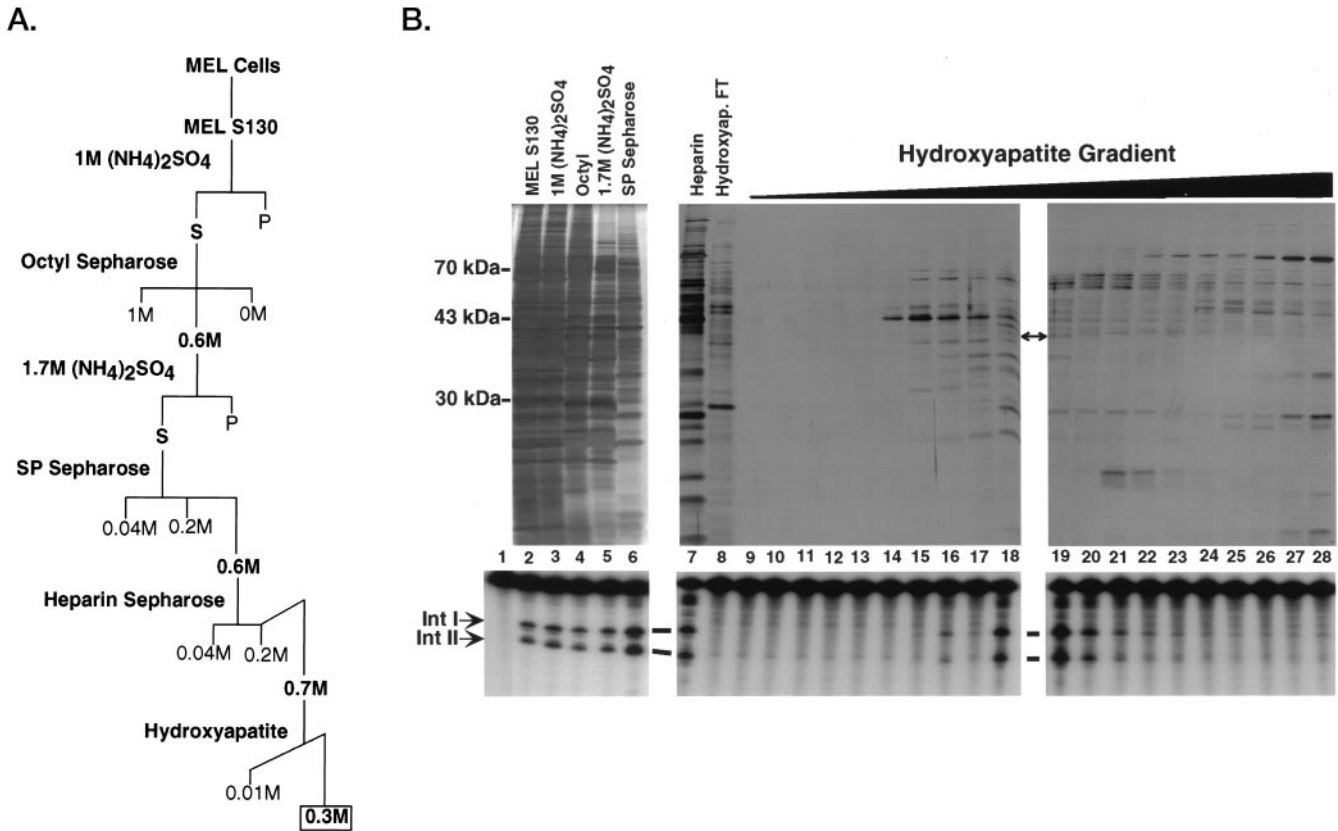


FIG. 4. **Partial purification of ErEN.** *A*, the purification scheme is outlined with the columns and fractionation listed on the left. Diagonal lines indicate an elution gradient. The fractions containing ErEN activity are in *bold type*. The final gradient resulting in the partially pure protein is boxed at the bottom. *B*, proteins from the active fractions during the purification were separated by SDS-PAGE and visualized by silver staining as labeled and shown in the top panel. The fractions from the hydroxyapatite gradient eluted with increasing concentration of Na_2HPO_4 ranging from 10 to 300 mM are shown (lanes 9–28). A candidate band corresponding to ErEN activity is indicated by the double arrow. Protein size markers are shown on the left in kilodaltons. The bottom panel is an autoradiograph of ErEN activity present in each fraction using uniformly labeled $\alpha 3'$ -UTR as substrate. The positions of the ErEN cleavage intermediates are labeled as in Fig. 2A.

with an increasing KCl gradient up to 0.7 M KCl. Active fractions were pooled, dialyzed in a buffer containing 10 mM Na_2HPO_4 (lane 7), bound to a hydroxyapatite column, and eluted with a Na_2HPO_4 gradient. Fractions from each purification step were resolved on SDS-PAGE and visualized by silver staining (top panel in Fig. 4B). Activity assays were also carried out on each fraction after dialysis (bottom panel of Fig. 4B). As seen in Fig. 4B, ErEN activity peaks in lanes 18 and 19. Only one candidate protein, whose appearance corresponds to ErEN activity, at ~40 kDa size range or smaller, could be identified. The putative ErEN protein is indicated by an arrow in Fig. 4B. Interestingly, neither with complete S130 extract (Figs. 1–3) nor with the purified fractions (Fig. 4) could complete cleavage of the input RNA be achieved. These data suggest that in addition to a sequence requirement, ErEN might also recognize a specific structural component within the MERCS and only a subset of the RNA generated *in vitro* form such a structure and are cleaved by ErEN.

Characterization of ErEN Cleavage—To begin a more thorough characterization of ErEN activity, we determined optimal parameters and cofactor requirements for ErEN cleavage. *In vitro* decay assays were carried out using uniformly labeled $\alpha 3'$ -UTR as the substrate. We initially determined the salt optimum by varying the concentration of KOAc (Fig. 5A, lanes 2–7). The highest amount of cleavage detected with partially pure protein from the heparin fraction was obtained at 100 mM KOAc (lane 4) as determined by the accumulation of the 5' cleavage product, Int I, and the 3' cleavage product, Int II. ErEN activity was inhibited by salt concentrations greater than 400 mM (lanes 6 and 7).

The dependence of ErEN activity on temperature was next tested. The decay reactions were carried out with protein from the heparin fraction in standard buffer conditions with 100 mM KOAc at various temperatures ranging from 4 to 55 °C (Fig. 5B). The greatest level of cleavage was detected at 37 and 45 °C (lanes 5 and 6, respectively). ErEN activity decreased above 45 °C although it could still be detected at 55 °C indicating that ErEN is a stable enzyme (lane 7). Collectively, our data indicate that ErEN is most active at salt and temperature parameters which are most biologically relevant.

Cofactor dependence for ErEN activity was also tested. We previously demonstrated that the addition of EDTA to 5 mM was sufficient to prevent exoribonucleolytic degradation of the ErEN cleavage intermediates but did not effect ErEN activity (30). These data suggested that ErEN did not require cations, however, it was not known whether ErEN cleavage could be enhanced or inhibited by different cations. Protein from the octyl-Sepharose fraction was used in these assays since it was devoid of any cations. The activity of ErEN in the presence of EDTA is shown in Fig. 5C, lane 2. EDTA was excluded from the reactions where different cations were tested (lanes 3–6). Neither the addition of Mg^{2+} nor Ca^{2+} appear to effect ErEN cleavage (lanes 3 and 5), however, both Mn^{2+} and Zn^{2+} alter ErEN activity (lanes 4 and 6). Mn^{2+} seems to partially inhibit ErEN activity (lane 4). Curiously, addition of Zn^{2+} completely inhibits ErEN cleavage (lane 6). Since the amount of the full-length $\alpha 3'$ -UTR probe does not appear to be effected by either Mn^{2+} or Zn^{2+} it seems unlikely that these cations increase exoribonuclease activity which would more efficiently degrade the ErEN intermediates. A more likely explanation is that

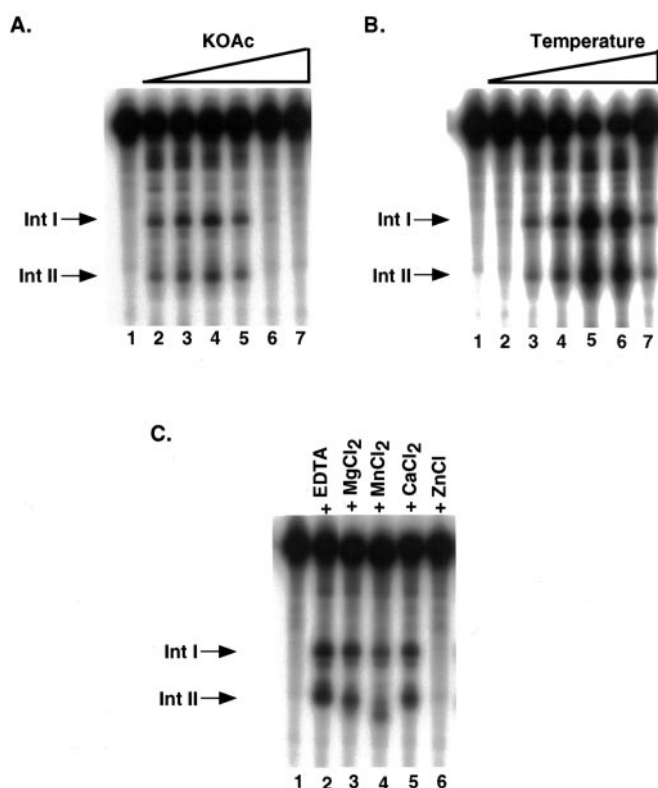


FIG. 5. **Characterization of ErEN cleavage.** *A*, *in vitro* decay assays were carried out using uniformly labeled $\alpha 3'$ -UTR and partially pure protein from the heparin fraction. *Lane 1* shows the migration of the input $\alpha 3'$ -UTR. *Lanes 2-7* show ErEN activity in the presence of increasing concentrations of salt corresponding to 25, 50, 100, 200, 400, and 600 mM KOAc. ErEN cleavage intermediates are as labeled in Fig. 2A. *B*, decay assays were carried out to determine the optimal temperature for ErEN cleavage. Uniformly labeled $\alpha 3'$ -UTR was incubated with protein from the heparin fraction at 4, 15, 25, 37, 45, and 55 °C in *lanes 2-7*, respectively. *C*, decay assays using $\alpha 3'$ -UTR uniformly labeled RNA and the octyl-Sepharose fraction in a buffer lacking cations was used to test the requirement of ErEN cleavage for cations. The position of input RNA is shown in *lane 1*. Cleavage in the presence of 5 mM EDTA is shown in *lane 2*. The reactions in *lanes 3-6* do not contain EDTA and include 2 mM of the indicated cation.

these two cations are inhibiting ErEN activity directly.

The nature of ErEN cleavage was next assessed by determining whether the cleavage step generated a 3' phosphate or a 3' hydroxyl. To address this we took advantage of the fact that polymerase can only attach a nucleotide to a 3' hydroxyl and not to a 3' phosphate. Uniformly labeled $\alpha 3'$ -UTR was cleaved by partially purified ErEN and then annealed to two different oligonucleotides complementary to the RNA. The first oligonucleotide was complementary to the last 18 nucleotides of the $\alpha 3'$ -UTR and contained an additional 25 nucleotides of uncomplementary sequence. The second oligonucleotide was complementary to the 3' end of Int I and then contained 16 random nucleotides. Both fragments were annealed to the ErEN-cleaved RNA and subsequently filled in with the Klenow fragment of DNA polymerase. Both the full-length and Int II were elongated 25 nucleotides by the polymerase as expected (Fig. 6, *lane 4*). However, a similar elongation was not detected with the oligonucleotide specific for Int I implying that ErEN does not generate a 3' hydroxyl but rather generates a 3' phosphate.

ErEN Is Contained Within a Protein Complex—During the purification of ErEN we observed that the pooled fractions containing ErEN activity off of the heparin column gave cleavage products with a slightly different mobility than those obtained using S130 extract or any of the purification steps prior

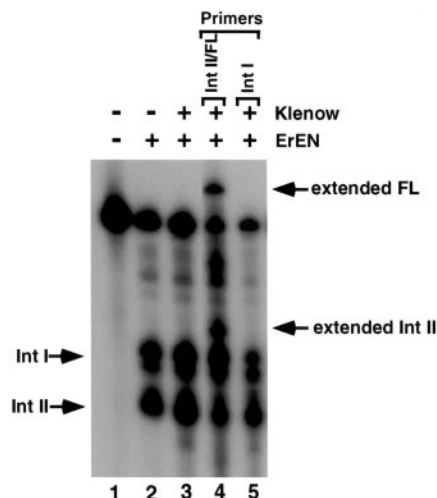


FIG. 6. **ErEN cleavage produces a 3' phosphate.** Uniformly labeled $\alpha 3'$ -UTR that had been cleaved by ErEN was annealed to specific primers and elongated using the Klenow enzyme. Only RNA containing a 3' OH can be utilized as a substrate for the Klenow mediated fill-in reaction. Incubation of the $\alpha 3'$ -UTR with Klenow enzyme is shown in *lane 3*. Extension reactions using a specific primer (Int II/FL) complementary to the 3' end of the RNA is shown in *lane 4*. This primer also contains 25 nt of noncomplementary sequence. Products that resulted from elongation by the polymerase in *lane 4* are labeled as extended full-length and extended Int II on the right. Klenow was unable to extend the 5' ErEN cleavage product (Int I) when a primer complementary to the 3' end of Int I was used (*lane 5*). This primer would extend the RNA by 16 nt. The lack of extension indicates the Int I RNA does not contain a 3' OH and therefore contains a 3' phosphate.

to the heparin step. A comparison of ErEN activity from MEL S130 to that of the heparin gradient was carried out in decay assays using the $\alpha 3'$ -UTR RNA as the substrate. The products of these reactions were separated on a sequencing polyacrylamide gel to detect single nucleotide differences in the product sizes. As seen in Fig. 7A, the cleavage products obtained from incubation with S130 extract are shown in *lane 2* and labeled Int I and Int II. These products correspond to cleavage at nucleotide 63 of the $\alpha 3'$ -UTR (Fig. 1A). The cleavage products resulting from incubation with the heparin-Sepharose eluate are shown in *lane 3* and labeled Hep Int I and Hep Int II. Based on comparison with the RNA ladder (*lane 4*), it appears that the cleavage with the heparin protein is occurring 2 to 3 nt downstream of the usual cleavage site. Interestingly, there is a CU at position 66 within the $\alpha 3'$ -UTR (Fig. 1A) and it appears that the cleavage is occurring at this position rather than at the CU at position 63. Furthermore, additional aberrant cleavages are routinely detected with the purified fractions (compare the top of *lane 2* to 3, and data not shown). These results suggest that additional factors are involved in the precise sequence specificity of ErEN which are present in S130 extract but lost during the purification.

The above results imply that ErEN is contained within a complex which increases the fidelity of ErEN cleavage. To determine whether ErEN is contained within a protein complex, gel filtration assays were carried out under nondenaturing conditions. MEL S130 extract that had been treated with micrococcal nuclease to disrupt any RNA-RNA bridging interactions, was resolved on a gel filtration column and the resulting fractions were assayed for ErEN activity (Fig. 7B). The peak of ErEN activity occurs at ~160,000 relative to the molecular weight standards suggesting that in total extract ErEN is associated with other proteins. Based on the size of this complex, it is possible that ErEN is in a complex with one or more proteins. Although ErEN can function on its own (Fig. 3), these additional proteins may regulate and provide further

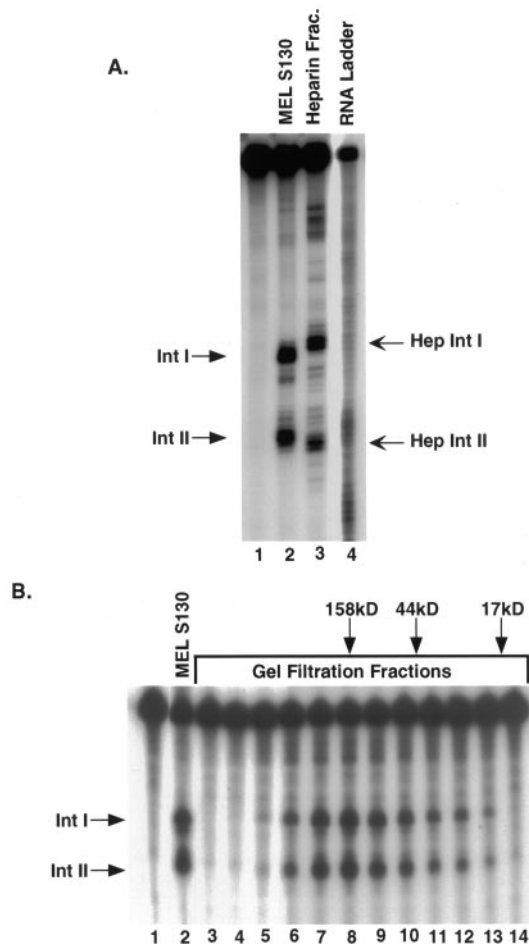


FIG. 7. **ErEN is a component of an ~160 kDa complex.** A, the products of decay reactions carried out with either MEL S130 extract (lane 2) or purified protein from the heparin fraction (lane 3) were resolved on an 8% denaturing sequencing gel to distinguish single nucleotide differences. Migration of the full-length $\alpha 3'$ -UTR is shown in lane 1. An alkaline RNA ladder is shown in lane 4 to identify single nucleotides. The intermediates seen with MEL S130 extract are labeled on the left and the intermediates detected with the heparin protein fraction are labeled on the right. B, gel filtration under native conditions was carried out using MEL S130 extract. The activity assay using $\alpha 3'$ -UTR as the substrate from the column fractions are shown in lanes 3–14. The activity of complete MEL S130 extract is shown in lane 2. The sizes of the molecular mass standards are shown above the bracket in kilodaltons.

specificity to ErEN activity. We cannot rule out the possibility that the ErEN protein functions as a tetramer although if that were the case it would be difficult to explain why the cleavage of the $\alpha 3'$ -UTR is altered upon purification of the protein.

DISCUSSION

In this report, we have mapped the MERCS within the $\alpha 3'$ -UTR required for cleavage by ErEN and presented data on our ongoing efforts to purify ErEN. We identified a 26-nt MERCS within the $\alpha 3'$ -UTR that is necessary and sufficient for ErEN recognition and cleavage (Fig. 1C). Substitution mutations throughout this region resulted in complete inhibition of ErEN cleavage (Fig. 2B) suggesting that this entire sequence is important for ErEN cleavage. However, this was somewhat surprising since not all of the mutations within the full-length $\alpha 3'$ -UTR spanning this 26-nt region effected ErEN cleavage, particularly the H23 mutation (Fig. 2A). Although the H19 and H21 mutations fall within the MERCS, neither inhibited ErEN activity completely. It is likely that ErEN recognizes a CU-rich sequence since 22 out of the 26 nt required for ErEN cleavage

are pyrimidines. The *Hind*III sites generated in the H19, H21, and H23 mutations retain a CUU sequence which may allow for ErEN recognition of these substrates. However, the more severe GAGAGA mutations at the same positions completely inhibit ErEN cleavage activity and appear to be drastic enough changes so that the RNA is no longer recognized as a substrate for ErEN. Once the gene encoding the ErEN protein is cloned we will be able to generate mutants which should allow us to distinguish between which sequences are required for cleavage and which are required for binding to the MERCS. The fact that the H19 and H21 mutations within the full-length $\alpha 3'$ -UTR change the specificity of ErEN cleavage is interesting and suggests that ErEN cleavage is somewhat flexible and may choose secondary cleavage sites if the optimal site is unavailable. This could suggest that ErEN might have more pleiotropic effects on RNAs in addition to the α -globin mRNA which are possibly regulated by the interacting proteins.

It has recently been shown that an mRNP stability complex forms on the β -globin 3'-UTR ($\beta 3'$ -UTR) and a component of this complex is α CP (43). The α CP proteins have been previously shown to form the α -complex on the $\alpha 3'$ -UTR (44, 45) suggesting that these two highly stable mRNAs may share some components involved in their stabilization. However, we have demonstrated that under our standard cleavage conditions the $\beta 3'$ -UTR is not a substrate for ErEN cleavage (30). It remains to be determined whether ErEN is able to cleave the β -globin mRNA under different conditions or at a position located within the 5'-UTR or the coding sequence.

ErEN cleavage does not require the presence of cations for its activity (Fig. 5C), particularly magnesium, which is often a required cofactor of nucleases. Similar to ErEN, endoribonucleolytic cleavage of the hepatitis B virus and albumin mRNAs is also independent of magnesium although PMR-1 activity is enhanced in the presence of magnesium (31, 33). It is also interesting that ErEN cleavage is inhibited in the presence of zinc, however, the significance of this is unclear at this time. Curiously, we have not been able to find conditions where ErEN could cleave all of the input RNA. This is also true with the purified fractions which could indicate that a particular RNA structure is also involved in recognition and cleavage by ErEN and only a subset of the *in vitro* generated RNA contain such a structure.

We have shown that under denaturing gel filtration conditions the size of ErEN is ~40 kDa (Fig. 3). One interesting outcome of our studies has been the demonstration that ErEN is in a protein complex of ~160 kDa as determined by gel filtration under native conditions (Fig. 7B). This suggests that in complete extract and most likely in cells, ErEN exists as a component of a multiprotein complex. We have also found that upon increased purity of ErEN, its normally tight sequence specificity is compromised and it begins to cleave more promiscuously (Fig. 7A).² Furthermore, fractions from the denaturing gel filtration column when resolved on a sequencing gel also contain similarly altered cleavage products (data not shown). Collectively, these data suggest that ErEN is normally found in association with one or more proteins which are most likely involved in maintaining the specificity of ErEN cleavage. It is interesting that ErEN is still able to recognize the $\alpha 3'$ -UTR on its own since cleavage still occurs after gel filtration in denaturing conditions and after partial purification of the protein. This suggests that the proteins that provide ErEN with sequence specificity may hold the enzyme or RNA in a particular conformation so that it cleaves only at nucleotide 63 within the

² N. D. Rodgers, Z. Wang, and M. Kiledjian, unpublished observations.

α 3'-UTR but may not be involved in recognition of the RNA target. When these additional proteins are absent, ErEN is still able to identify substrate RNAs and may be in a conformation such that it is able to cleave the RNA at different positions.

The fact that ErEN appears to be in a protein complex also suggests that the other proteins in the complex may regulate ErEN activity under certain conditions. Recently, Cunningham *et al.* (47) have demonstrated that PMR-1 is held in a large protein complex in an inactive form until estrogen activates a signal transduction pathway which eventually releases the inhibition of PMR-1 such that it cleaves the albumin mRNA. It is possible that a similar scenario may be true for ErEN. ErEN could be in one activity state under certain conditions and another upon cell differentiation or external stimulus. This also raises the possibility that ErEN may be present in other cell types but remains inactive until specific conditions change. It is conceivable that if ErEN is expressed in other cell types it may target mRNAs containing a similar CU-rich region (42, 48–54). Once the ErEN protein is purified and cloned it will be interesting to determine other RNA substrates, the pattern of expression as well as the components that make up the detected ~160-kDa protein complex, and to determine how these proteins regulate ErEN activity.

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