Physical evidence supporting a ribosomal shunting mechanism of translation initiation for BACE1 mRNA

Dora C. Koh, Gerald M. Edelman and Vincent P. Mauro*

Department of Cell and Molecular Biology; The Scripps Research Institute; La Jolla, CA USA

*Current Affiliation: Promosome, LLC; San Diego, CA USA

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Cleavage of the Amyloid Precursor Protein (APP) by cellular proteases gives rise to various peptides, including a set of 38–43 amino acid peptides (Aβ) that are generated when APP is cleaved by the β-site APP cleaving enzyme 1 (BACE1) and γ-secretase.1,2 Although these peptides are generated normally, different factors including elevated BACE1 levels, can lead to increased levels of Aβ peptides in brain and have been postulated to cause Alzheimer disease pathology.3 Various studies have indicated that increased BACE1 levels are not the result of increases in its mRNA levels, but rather involve post-transcriptional mechanisms including translation.4

Translation of rat and human BACE1 mRNAs is 5’ cap-dependent and does not appear to involve internal initiation of translation.5,7 In addition, although the BACE1 5’ leader contains 4 AUG codons, various studies have shown that mutation of these codons had either relatively small effects on translation or no effect, suggesting that they are not used as translation start sites.5,8 By contrast, other studies reported that translation initiation at upstream AUG codons had significant inhibitory effects on translation.6,7 We showed that some of these differences were caused by the expression systems.9 Reporter mRNAs initiated translation more often at upstream AUG codons when in vitro transcripts were transfected into cells, or when mRNAs were transcribed in the cytoplasm. However, comparable mRNAs initiated translation predominantly at the initiation codon when transcribed in the nucleus. We have suggested that these various observations are more consistent with a mechanism in which ribosomal subunits bypass segments of the 5’ leader as they move from the cap to the initiation codon. This is inconsistent with a scanning-based mechanism of translation initiation.5,9

In this study, we looked for physical evidence of the BACE1 translation mechanism by using lead(II) acetate to probe the structural stability of endogenous BACE1 mRNAs in live cells. Pb2+ is cell permeable and induces RNA cleavage that is...
RNA was chosen as an exemplar inasmuch as it has been studied extensively and various data indicate that specific structural features in this RNA are universally conserved. For this analysis, B104 cells were incubated with different concentrations of lead(II) acetate. The Pb²⁺-treated cells excluded trypan blue dye in a degree comparable to that of control cells, indicating that they remained alive during treatment. RNA was extracted from the cells and an indication of the extent of cleavage was obtained by monitoring the integrity of the rRNA bands in agarose gels. It was found that the extent of cleavage depends on the exposure time and concentration of lead(II) acetate (Fig. 1A). Primer extension analysis (Fig. 1B, C) revealed a major band corresponding to the full-length RNase P RNA in control samples for each primer (0 mM Pb²⁺; see arrow). The lower molecular weight bands are likely to correspond to premature stops by the reverse transcriptase enzyme or represent extension products from fragmented templates. Exposing cells to lead(II) acetate led to degradation of the full-length RNase P RNA and the appearance of new bands corresponding to cleavage sites.

To avoid secondary cuts, we sought to cleave target RNAs less than once per molecule on average. Suitable conditions were obtained by incubating cells with up to 100 mM lead(II) acetate for 15 min at 37°C. Secondary cuts appeared to be minimal or of no consequence because no new bands were observed when cells were exposed to the highest Pb²⁺ concentration (100 mM) as compared with the lowest concentration (25 mM).

This chemical is particularly well suited for the present efforts inasmuch as the coding region of an actively translated mRNA is continuously destabilized by ribosomes and should have sufficient conformational flexibility to be an ideal substrate for Pb²⁺-induced cleavage. The extent of cleavage in the 5’ leader should therefore provide an indication of the translation mechanism. For example, ribosomal scanning of the 5’ leader from the cap to the initiation codon, followed by translation of the coding region should fully destabilize both regions with each round of translation. By contrast, if segments in the 5’ leader are not destabilized compared with the coding region, this would suggest that they are bypassed as ribosomal subunits move from the cap to the initiation codon.

Results

Assessing feasibility of lead probing in mammalian cells. Inasmuch as lead(II) acetate has not been used previously to probe RNA structures in mammalian cells, we first assessed the feasibility of our methodology by probing RNase P RNA. This RNA was chosen as an exemplar inasmuch as it has been studied extensively and various data indicate that specific structural features in this RNA are universally conserved. For this analysis, B104 cells were incubated with different concentrations of lead(II) acetate. The Pb²⁺-treated cells excluded trypan blue dye in a degree comparable to that of control cells, indicating that they remained alive during treatment. RNA was extracted from the cells and an indication of the extent of cleavage was obtained by monitoring the integrity of the rRNA bands in agarose gels. It was found that the extent of cleavage depends on the exposure time and concentration of lead(II) acetate (Fig. 1A). Primer extension analysis (Fig. 1B, C) revealed a major band corresponding to the full-length RNase P RNA in control samples for each primer (0 mM Pb²⁺; see arrow). The lower molecular weight bands are likely to correspond to premature stops by the reverse transcriptase enzyme or represent extension products from fragmented templates. Exposing cells to lead(II) acetate led to degradation of the full-length RNase P RNA and the appearance of new bands corresponding to cleavage sites. To avoid secondary cuts, we sought to cleave target RNAs less than once per molecule on average. Suitable conditions were obtained by incubating cells with up to 100 mM lead(II) acetate for 15 min at 37°C. Secondary cuts appeared to be minimal or of no consequence because no new bands were observed when cells were exposed to the highest Pb²⁺ concentration (100 mM) as compared with the lowest concentration (25 mM).
The results showed that most of the primer P1 extension products had bands corresponding to those from other primers (Fig. 1C), suggesting that the observed extension products represented authentic cleavage sites and not products generated from spuriously primed RNA templates. Both strong and weak bands were observed within each primer extension reaction. In addition, the relative intensities of some bands varied with different primers. These differences may be related to the ability of some cleaved fragments to adopt altered conformations that allow hybridization to various primers with different efficiencies. By mapping the major sites of cleavage onto a previously predicted secondary structure of the RNase P RNA, it can be seen that the cleavage sites are consistent with various previously predicted structural features (Fig. 1D). For example, cleavage sites provide physical evidence for the occurrence of paired regions 10/11 (P10/11), conserved region II (CRII) and CRIII, and paired region P12, demonstrating the validity of this method in vivo.

Subcellular distribution of BACE1 mRNAs in cells. Investigations of RNA structural features in vivo may be complicated by the fact that RNAs do not necessarily occur as homogeneous populations within cells. Indeed, mRNA structures in the cytoplasm may differ significantly from those in the nucleus, particularly mRNAs that undergo significant processing in the nucleus and interact with different factors in each location. In the cytoplasm, translation is expected to disrupt secondary structures in coding regions. Whether translation also occurs in the nucleus remains unresolved; nevertheless, it is important to determine how an mRNA is distributed within cells to understand whether the Pb²⁺-probing results reflect cleavage of mRNAs that are primarily localized to the cytoplasm, nucleus, or present at a significant level in both compartments. To determine the subcellular distribution of BACE1 mRNAs, RNA was isolated from the nuclei or cytoplasm of rat PC12 and B104 cells and RNase protection assays (RPAs) were performed using a radiolabeled probe specific to the BACE1 mRNA. We observed that BACE1 mRNA levels are significantly lower in B104 than PC12 cells, and that this mRNA is found almost exclusively in the cytoplasm (Fig. 2A). This finding indicates that any cleavage sites mapped in the Pb²⁺-cleavage experiments are derived from cytoplasmic BACE1 mRNAs. To ensure that RNA was present in the nuclear fraction, we used nuclear and cytoplasmic fractionation for RNase P RNA, and showed that this RNA was present in both nuclear and cytoplasmic fractions as previously reported. 20

Another potential source of heterogeneity in the BACE1 mRNA population arises from translation itself. If a subset of BACE1 mRNAs is not translated or if some mRNAs are translated with different efficiencies, then different mRNAs in the population may have different structural features. To investigate this potential source of heterogeneity, cell lysates were fractionated in sucrose density gradients and individual fractions were hybridized with radiolabeled probes for BACE1 mRNA (Fig. 2B). The polysome profiles show that this mRNA is localized in polysomes and is not detected in 80S monosomes or lighter fractions. The data indicate that BACE1 mRNAs are associated with several ribosomes, suggesting that this mRNA population is relatively uniform in its translation properties. This finding is consistent with our earlier finding that the BACE1 5' leader mediates relatively efficient translation. 5

Lead probing of endogenous BACE1 mRNAs in cells. Lead(II) acetate probing of endogenous BACE1 mRNA was performed in PC12 and B104 cells using the approach described above for RNase P RNA. Due to the low signals obtained with B104 cells, it was difficult to achieve complete coverage of the 5' leader with at least 2 primers, even when reactions were scaled up. We therefore pursued this analysis in PC12 cells. Suitable conditions for Pb²⁺-induced RNA cleavage in PC12 cells (Fig. 3A) were similar to those in B104 cells (Fig. 1A). Primer extension reactions were performed using primers complementary to various sites in the BACE1 mRNA (Fig. 3B). Data from 3 primers which show the Pb²⁺ cleavage pattern throughout the BACE1 5' leader, extending into the coding region, are presented in Figure 3C. The results reveal two primer extension products containing 441 or 445-nucleotides that appear to correspond to the 5' end of the BACE1 mRNA (indicated by arrows in Figure 3C). These bands are respectively 14 and 18-nucleotides longer than the 427-nt cDNA sequence used in our earlier studies (see asterisk; 5,9), and are similar in length to human BACE1 5' leader sequences that comprise 446–457 nucleotides. 21

Cleavage sites that were observed with 2 or more primers were plotted on a linear representation of the BACE1 5' leader and start of the coding region (Fig. 4A). Nucleotides downstream of the 5' end for more than a 100-nucleotide length were highly resistant to cleavage. A few cleavage sites were observed further downstream, starting in the vicinity of the second upstream
≈150 nucleotides and extended into the coding region. Cleavage sites did not appear to coincide with any of the upstream AUG codons. We compared this data to the restricted data obtained from B104 cells and found that the cleavage patterns are almost identical, particularly when cleavage sites from single primers were included for comparison (Fig. S1).

**Sequence analysis of BACE1 5' leader.** To investigate whether the Pb⁺⁺ cleavage patterns observed in our experiments might be explained partially or fully by inherent differences in the ability of the BACE1 mRNA to form secondary structures in different parts of the mRNA, we compared the Pb⁺⁺ cleavage data to predicted stabilities in these regions of the mRNA. Predicted stabilities along the mRNA were calculated as minimum free energies using the RNA structure prediction program RNAfold (Fig. 4B;22). Stabilities were calculated using a sliding window of 30-nucleotides, walking 5'→3' in 10-nt steps. For reference, the minimum free energy of an unstructured segment of RNA is 0 kcal/mol, and that of a structured segment is a negative number that becomes increasingly negative with increased stability. The results predict regions of high and low structural stability, but in general, the cleavage sites do not correspond to segments in the mRNA that have low predicted stability. For example, the region upstream of the initiation codon has relatively high predicted stability, but is highly susceptible to cleavage, whereas some segments located further upstream are predicted to be less structured, but are resistant to cleavage.

To further analyze the BACE1 5' leader, we looked more closely at its nucleotide composition (Fig. 4C). We observed that both the 5' leader and start of the coding sequence are rich in guanine and cytosine nucleotides (> 60% GC) and that the distribution of these nucleotides is relatively uniform across this region. By contrast, the distribution of adenine and uracil nucleotides is less uniform in the region surrounding the initiation codon (the arrow indicates the start of the coding region). Adenine nucleotides are more abundant upstream of the initiation codon than downstream and U has the opposite distribution. Although this nucleotide distribution appears to define a boundary between the 5' leader and the coding region, there is no direct correlation between this distribution, the pattern of Pb⁺⁺-induced cleavage (Fig. 4A), or the predicted stabilities (Fig. 4B).

**Determining the effects of the eIF4A helicase on BACE1 structure.** Earlier studies indicated that translation initiated via the BACE1 5' leader is cap-dependent.5-7 This suggests that ribosomal recruitment by this mRNA involves the eIF4F cap-binding complex. An important component of this complex is initiation factor eIF4A, an ATP-dependent non-processive RNA helicase.23,24 To investigate whether the extensive lead-induced cleavage observed upstream of the initiation codon might be due in part to destabilization of this region by the helicase activity of this initiation factor, we inhibited eIF4A activity using hippuristanol, a small molecule that is a selective and potent inhibitor of eIF4A RNA-binding activity.25 The results of 35S-Met labeling revealed that overall protein synthesis was inhibited by treating cells with increasing concentrations of hippuristanol. We observed that exposure of PC12 cells to 0.5 μM hippuristanol for 50 min decreased overall protein synthesis by 90%, and by

![Figure 3](image-url)
95% with 50 μM hippuristanol. To determine whether blocking eIF4A activity affects the structure of the BACE1 5’ leader, we probed endogenous BACE1 mRNAs with lead(II) acetate in hippuristanol-treated cells (Fig. S2). Surprisingly, only a few minor changes were observed, even after up to 8 h of treatment. Changes included the loss of some cleavage sites and the appearance of additional bands in the sample without Pb2+ (0 mM). However, the overall cleavage pattern was virtually unchanged from that in untreated control cells. This finding suggests that the structure of the BACE1 mRNA is largely unaffected by the helicase activity of eIF4A.

**Discussion**

For this work, we investigated the translation mechanism of BACE1 mRNA by probing the structural stability of the 5’ leader of endogenous BACE1 mRNAs in cells using lead(II) acetate. The ability of Pb2+ to induce cleavage of single stranded RNA with conformational flexibility made it ideal for assessing whether the BACE1 5’ leader is destabilized in vivo, consistent with a scanning mechanism, or whether the 5’ leader or parts of it remain structured, consistent with a shunting mechanism.

The Pb2+-probing results obtained for BACE1 mRNA indicate that the first ∼280 nucleotides of the 5’ leader are resistant to Pb2+-induced cleavage. The remainder of the 5’ leader (∼150 nucleotides) and start of the coding region are susceptible to cleavage. Cleavage in the coding region was anticipated, as this region is destabilized by ribosomes in the elongation phase of protein synthesis. However, the cleavage pattern in the 5’ leader is less obvious. The finding that the 5’ end of the mRNA is resistant to cleavage was somewhat unexpected as BACE1 translation appears to be cap-dependent, and it was thought that the helicase activity associated with the eIF4F complex, which is bound to the cap-structure, would destabilize RNA structures near the cap and render the 5’ end of the mRNA susceptible to cleavage. This does not appear to be the case indicating that the helicase activity of the cap-binding complex does not destabilize the BACE1 mRNA near the cap structure.

The non-uniform cleavage of the BACE1 5’ leader suggests that ribosomal subunits recruited at the 5’ cap-structure bypass a large segment of the 5’ leader to reach the initiation codon. Our earlier studies using BACE1reporter constructs supported a similar conclusion.5,9 This cleavage pattern is not consistent with the scanning mechanism of initiation26–27 which proposes that ribosomal subunits destabilize and inspect the 5’ leader, base-by-base, until they recognize a start site. If each round of elongation involves a round of scanning, both 5’ leader and coding region should be equally destabilized in each round. This reasoning also applies to leaky scanning and reinitiation, which are variations of the scanning model. Nevertheless, we can imagine several possible scenarios whereby scanning might explain the data. For example, if scanning is significantly faster than elongation, the 5’ leader might restabilize before cleavage can occur. However, this possibility seems unlikely as experiments performed in yeast have estimated the rate of scanning to be ∼10-nucleotides/second based on time differences observed for translation of mRNAs with 5’ leaders of different lengths.28 This estimate is not significantly different than the estimated rate of elongation, which is ∼9–24-nucleotides/second.29 Another possible scenario is that BACE1 mRNAs comprise structurally and functionally heterogeneous mRNA populations, and the Pb2+ probing results reflect cleavage of subpopulations of BACE1 mRNAs, some of which are highly structured and poorly translated. We cannot rule out this possibility completely, but it seems unlikely as we have shown that most BACE1 mRNAs are located in the cytoplasm and reside in large polysomes (Fig. 2). It is possible that these large polysomes contain stalled complexes. However, this possibility is also considered unlikely as large BACE1 polysomes are consistent with previous findings indicating that translation mediated by the BACE1 5’ leader is relatively efficient as assessed by comparison with a highly efficient reference 5’ leader.3

The extensive cleavage observed upstream of the BACE1 initiation codon may be due in part to native secondary structure adopted by the 5’ leader. This notion seems unlikely at first glance, given the high, relatively uniform GC content of this 5’ leader and its potential to adopt a secondary structure. However, the A-U composition upstream of the start site is biased in a way that may decrease stability in this region. This bias—toward A and
This yeast study examined mRNAs containing 5' leaders of 100 or more nucleotides with translation start sites that had been established with a high level of confidence. They found that nucleotide frequencies are fairly constant throughout 5' leaders, except for the region ~40-nucleotides upstream of initiation codons, where the frequency of A increases and that of U decreases. The authors postulated that this distribution suggests a selection for reduced RNA structure inasmuch as the base pairing potential of A (A:U) is less than that of U (U:A:U:G). The nucleotide distribution in the BACE1 5’ leader suggests that the region upstream of the initiation codon for ~80 nucleotides (downstream of uORF3) may be less structured than other parts of the 5’ leader. However, cleavage continues for another ~70 nucleotides upstream of this region.

A possible explanation for why the upstream region is susceptible to cleavage is that it is actively destabilized by the translation of a uORF. Although the cleavage pattern does not line up with any of the uORFs, it does correspond to a CUG codon in good nucleotide context that is located 10-nucleotides downstream of where cleavage becomes relatively uniform. The CUG-initiated ORF extends 33-nucleotides before it overlaps uORF3 in the same reading frame, generating a 54-nt ORF. Initiation at this CUG codon might explain the observed cleavage pattern from ~80–150 nucleotides upstream of the initiation codon.

In an earlier study, we demonstrated that the relative accessibility of AUG can affect how efficiently it functions as an initiation codon. If the intrinsic conformation of the BACE1 5’ leader predisposes the initiation codon to be more accessible than other potential start sites, this may help to determine the site of initiation for the first round of translation. This first round of translation should dramatically restructure the mRNA by destabilizing the coding region and bringing eIF4A to the vicinity of the initiation codon. We initially expected that the helicase activity of eIF4A might contribute to the accessibility observed upstream of the start site and increase the likelihood of subsequent initiation events. This did not appear to be the case: the cleavage pattern was essentially unaffected upon blocking eIF4A with hippuristanol. At present we cannot yet rule out the activities of other helicases.

The present study provides a glimpse of the conformation of endogenous BACE1 mRNAs in live cells. The results show that a large segment of the 5’ leader is not destabilized. This is consistent with a ribosomal shunting mechanism of translation initiation. In other work, this laboratory has studied ribosomal shunting using synthetic constructs and proposed two initiation mechanisms that involve tethering or clustering of the translation machinery. These mechanisms can account for nonlinear ribosomal movement during translation initiation and provide a plausible explanation for the BACE1 data.

We anticipate that improvements in the sensitivity of the Pb²⁺-probing method will facilitate future investigations of mRNA structure in postmortem human tissues in the future. The ability to perform such studies will enable a comparison of structural features in BACE1 mRNAs of normal and Alzheimer disease brains to determine whether increased BACE1 levels in the disease are accompanied by structural alterations in BACE1 mRNAs that may affect translation.

### Materials and Methods

**Cell culture and lead(II) acetate treatment.** Rat pheochromocytoma PC12 and neuroblastoma B104 cells were cultured using Dulbecco’s modified minimal essential medium (Gibco BRL, Life Technologies) supplemented with 10% fetal bovine serum (B104 cells) and 1% penicillin-streptomycin-glutamine in 10 cm tissue culture plates at 37°C in 5% CO₂. PC12 cells were supplemented with 5% fetal bovine and 5% horse serum and cultured on collagen coated plates. Cells were grown to 60–70% confluency and rinsed with phosphate buffered saline (PBS) prior to incubation with 3ml control buffer or lead(II) acetate trihydrate (Sigma Aldrich) solutions, which contained 10 mM TRIS-acetate (pH 7.5), 40 mM NaCl, and 5 mM MgCl₂. Cells were incubated at 37°C for up to 30 min as indicated. Reactions were quenched with a 1.5 molar excess of EDTA to chelate the lead. RNA was extracted using Trizol reagent (Invitrogen).

The extent of Pb²⁺-induced RNA cleavage was determined by extracting total RNA with Trizol reagent, treating with DNase (DNasefree kit; Ambion) and analyzing the RNA on denaturing agarose gels stained with ethidium bromide. Primer extension analyses were performed using cloned AMV reverse transcriptase (Life Technologies). Reactions contained 0.2 pmol of end-labeled PAGE purified primer added to 40 μg of DNase-treated total RNA from cells (final volume 7 μl). RNA/primer mixes were incubated at 85°C for 5 min and slowly cooled to 45°C prior to addition of other reagents. Extension products were RNase digested, purified and resolved on 8% sequencing gels (SequaGel-8; National Diagnostics). Positions of cleavage sites were determined using sequencing reactions from rat RΝase P and BACE1 genes that were electrophoresed alongside the primer extension reactions.

**RNA analysis.** For RNA extraction from nuclear and cytoplasmic fractions, cell media was aspirated and tissue culture plates placed on ice. Nuclear and cytoplasmic fractions were prepared according to ref. 35. RNA was extracted using Trizol reagent. The nuclear pellet was washed with nuclear resuspension buffer and centrifuged at 5,000 rpm at 4°C for 5 min. RNA was extracted from the pellet using Trizol reagent. Polysome analyses using sucrose density gradients and RPAs were performed as previously described.

**Primer sequences used for primer extension:**

- P1 [3’-CCA CTC CGT GGA GCC CCC GGA GTA TTG GG-5’];
- P2 [3’-GTT ACC GAC TCC ACT CCG TGG AGC GCC CGG-5’];
- P3 [3’-CAA GGG TCT CTC GTC CCG AGG TGC GCA CCC G-5’];
- P4 [3’-GAT TGG CCC GAG AGG GGC TCA CCC CTC CAC-5’];
- P5 [5’-GTC TTC CAG GCC GAC TTT GCA GCG TGA C-3’];
- P6 [5’-GCT CCC GCC GGT GGA GAG TGG GCA GCA GGA G-3’];
- P7 [5’-GAT ACC GAT AGT TCC CTG GGC AGG CAG CAG-3’].
Supernatants were used directly for trichloroacetic acid (TCA) precipitation. Protein samples were spotted in duplicate on filter discs and left to dry. Filters were then washed with room temperature 5% TCA for 3 min, hot 5% TCA for 4 min, followed by two washes with room temperature 5% TCA for 3 min and a 100% ethanol for 2 min. The filters were dried prior to scintillation counting. For lead probing experiments, cells were incubated with 0.5 μM hippuristanol for 6–8 h at 37°C prior to treatment with lead(II) acetate trihydrate solution. Control cells were incubated in media containing 0.5% DMSO.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental material may be found here (www.landesbioscience.com/journals/translation/article/24400)
36. Mathews DH, Sabina J, Zuker M, Turner DH. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol 1999; 288:911-40; PMID:10329189; http://dx.doi.org/10.1006/jmbi.1999.2700.