A Conserved cis-Acting Element in the Parathyroid Hormone 3'-Untranslated Region Is Sufficient for Regulation of RNA Stability by Calcium and Phosphate*

Rachel Kilav, Justin Silver, and Tally Naveh-Many‡

From the Minerva Center for Calcium and Bone Metabolism, Nephrology Services, Hadassah University Hospital, Jerusalem Il-91120, Israel

Calcium and phosphate regulate parathyroid hormone (PTH) gene expression post-translationally by changes in protein-PTH mRNA 3'-untranslated region (UTR) interactions, which determine PTH mRNA stability. We have identified the protein binding sequence in the PTH mRNA 3'-UTR and determined its functionality. The protein-binding element was identified by binding, competition, and antisense oligonucleotide interference. The sequence was preserved among species suggesting its importance. To study its functionality in the context of another RNA, a 63-base pair cDNA PTH sequence was fused to the growth hormone (GH) gene. There is no parathyroid (PT) cell line and therefore an in vitro degradation assay was used to determine the stability of transcripts for PTH, GH, and a chimeric GH-PTH 63 nucleotides with PT cytosolic proteins. The full-length PTH transcript was stabilized by PT proteins from rats fed a low calcium diet and destabilized by proteins from rats fed a low phosphate diet, correlating with PTH mRNA levels in vivo. These PT proteins did not affect the native GH transcript. However, the chimeric GH transcript was stabilized by low calcium PT proteins and destabilized by low phosphate PT proteins, similar to the PTH full-length transcript. Therefore, we have identified a PTH RNA-protein binding region and shown that it is sufficient to confer responsiveness to calcium and phosphate in a reporter gene. This defined element in the PTH mRNA 3'-UTR is necessary and sufficient for the regulation of PTH mRNA stability by calcium and phosphate.

Parathyroid hormone (PTH)³ acts to maintain serum calcium within a narrow physiological range (1). A 7-transmembrane branched calcium-sensing receptor on the parathyroid (PT) cell recognizes small changes in serum-ionized calcium and regulates PTH secretion (2). Low serum calcium increases PTH secretion, PTH mRNA levels (3), and if prolonged, PT cell proliferation (4). PTH then acts to correct serum calcium by mobilizing calcium from bone and increasing renal reabsorption of calcium. Phosphate also regulates the PT, with low serum phosphate decreasing serum PTH, PTH mRNA levels, and PT cell proliferation (4–8).

The effects of dietary calcium and phosphate on PTH gene expression are both post-transcriptional (5, 9). The PTH cDNA consists of three exons coding for the 5'-UTR (exon I), the prepro region of PTH (exon II), and the structural hormone together with the 3'-UTR (exon III) (10, 11). The rat 3'-UTR is 239 nt long out of the 712 nt of the full-length PTH RNA (11). The 3'-UTR is 42% conserved between human and rat, while the coding region is 78% conserved at the nt level (11).

We have shown that cytosolic proteins from PTs bind to the 3'-UTR of the rat PTH mRNA (9). PT proteins from hypocalcemic rats show increased binding to the PTH mRNA 3'-UTR by mobility shift and UV cross-linking assays and this protein-RNA binding is decreased with hypophosphatemic PT proteins. Thus the level of protein-RNA binding directly correlates with PTH mRNA levels. Since there is no PT cell line, an in vitro PTH RNA stability assay was utilized. This assay showed stabilization of the PTH transcript by hypocalcemic PT proteins and marked instability with PT hypophosphatemic proteins (9). A PTH transcript that did not include the 3'-UTR was not degraded by PT proteins in this assay. These studies indicate that there are instability regions in the PTH mRNA 3'-UTR that are protected by RNA-binding proteins. We have recently identified two proteins that bind the PTH mRNA 3'-UTR (12, 13). One protein, AUF1, was identified by affinity chromatography and was shown to stabilize the PTH transcript in an in vitro degradation assay with PT proteins (12). In other RNAs such as proto-oncogenes and cytokines, that have a short half-life, AUF1 is associated with destabilization of the transcripts (14). A second protein, dynein light chain (M, 8000) or LC8 was identified by expression cloning and was shown to mediate the binding of the PTH mRNA to microtubules (13). LC8 may have a role in the intracellular localization of PTH mRNA in the PT cell rather than in the stability of PTH mRNA.

We have now identified the minimal protein binding sequence in the PTH mRNA 3'-UTR by binding assays, competition experiments, and oligonucleotide binding interference analysis. To demonstrate the functionality of this sequence 63 base pairs of the PTH mRNA 3'-UTR which included the 26-nt protein-binding element and 5'- and 3'-flanking nt were inserted into the GH gene. Using in vitro degradation assays with cytosolic extracts from parathyroid glands we demonstrated that this element altered GH mRNA stability. The 63-nt element also reduced the stability of a random transcript of the pCRII polylinker, in contrast to a truncated element inserted into the polylinker which did not bind PT proteins. In addition,
inserting the 63-nt binding sequence into GH RNA conferred responsiveness of the chimeric transcript to PT proteins from low calcium and low phosphate rats, similar to the effects of these proteins on the full-length native PTH transcript. These results demonstrate that the protein-binding region in the PTH mRNA 3′-UTR is sufficient to determine PTH mRNA stability in response to changes in serum calcium and phosphate.

**EXPERIMENTAL PROCEDURES**

**Animals—**Weanling male Sabra rats were fed a normal calcium (0.6%), normal phosphate (0.3%) diet; or a low calcium (0.02%), normal phosphate (0.3%) diet; or a low phosphate (0.02%), normal calcium (0.6%) diet (Teklad, IL) for 3 weeks. At 3 weeks the thyroparathyroid tissue was removed under pentobarbital anesthesia and blood samples were taken for serum calcium and phosphate. The low calcium diet resulted in a serum calcium of 4.6 ± 0.4 mg/dl (control = 11.2 ± 0.3 mg/dl). The low phosphate diet resulted in a serum phosphate of 4.0 ± 0.6 mg/dl (control = 9.5 ± 0.7 mg/dl) and serum calcium of 12.4 ± 0.8 mg/dl.

**Cytoplasmic Protein Purification—**Cytoplasmic thyroparathyroid proteins (S100) for protein-RNA binding were extracted by a modification of the method of Dignam et al. (15). Tissues were removed from the rats and immediately washed in cold phosphate-buffered saline. The tissue was homogenized in 5 volumes of buffer A containing A (0.02 M) HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride, and incubated on ice for 10 min. After centrifugation at 10,000 g for 10 min (4 °C) the supernatant was centrifuged further at 100,000 g for 1 h (Beckman Type TL-100). For RNA degradation assays the S100 fraction was prepared as previously described (9) by homogenizing the tissue in 2 volumes of 10 mM Tris- HCl, pH 7.4, 0.5 mM DTT, 10 mM KCl, 1.5 mM MgCl₂, 0.1 Volume of the extraction buffer (1.5 mM KC1, 15 mM MgCl₂, 100 mM Tris-HCl, pH 7.4, 5 mM DTT, was added and the homogenate was centrifuged at 100,000 x g for 1 h at 4 °C. Cytoplasmic extracts were immediately frozen at −80 °C in aliquots. Protein concentration was determined by O.D. densitometry (0.95 μm wavelength) using a Bradford reagent (Bio-Rad).

**RNA Transcripts and Probes—**Labeled and unlabeled RNA was transcribed from linearized plasmids using an RNA production kit (Promega, WI) and the appropriate RNA polymerases. The specific activity of the RNA probe was 0.5–1.0 × 10⁹ cpm/μg. For competition experiments unlabeled RNA was transcribed similarly in the presence of 1 mM methylsulfonyl fluoride, and incubated on ice for 10 min. After centrifugation at 14,000 g for 3 min (4 °C) the supernatant was carefully collected. For the 30-nt sense oligonucleotide was ATTAACCCTCACTAAAG-GGACAATATATTCTTTTTTTAAAGTATTA, and the antisense oligonucleotide was TAATACGACTCACTATAGGG. For the 63-nt oligonucleotide was ATTAACACCCTACAG-GGACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 100-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 150-nt oligonucleotide was ATTAACCCAACGCAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 200-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. The specific activity of the RNA probe was 5 × 10⁶ cpm/μg. For competition experiments unlabeled RNA was transcribed similarly in the presence of 1 mM methylsulfonyl fluoride, and incubated on ice for 10 min. After centrifugation at 14,000 g for 3 min (4 °C) the supernatant was carefully collected. For the 30-nt sense oligonucleotide was ATTAACCCTCACTAAAG-GGACAATATATTCTTTTTTTAAAGTATTA, and the antisense oligonucleotide was TAATACGACTCACTATAGGG. For the 63-nt oligonucleotide was ATTAACACCCTACAG-GGACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 100-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 150-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 200-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 250-nt oligonucleotide was ATTAACCCCACCTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. The specific activity of the RNA probe was 5 × 10⁶ cpm/μg. For competition experiments unlabeled RNA was transcribed similarly in the presence of 1 mM methylsulfonyl fluoride, and incubated on ice for 10 min. After centrifugation at 14,000 g for 3 min (4 °C) the supernatant was carefully collected. For the 30-nt sense oligonucleotide was ATTAACCCTCACTAAAG-GGACAATATATTCTTTTTTTAAAGTATTA, and the antisense oligonucleotide was TAATACGACTCACTATAGGG. For the 63-nt oligonucleotide was ATTAACACCCTACAG-GGACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 100-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 150-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 200-nt oligonucleotide was ATTAACCCCACTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. For the 250-nt oligonucleotide was ATTAACCCCACCTACAGGG-GACATTTCCCCATTTTCAATAGATT, and the antisense oligonucleotide was ATTAATTTTAAAAAGGAGATATTTGAGATTG. The specific activity of the RNA probe was 5 × 10⁶ cpm/μg. For competition experiments unlabeled RNA was transcribed similarly in the presence of 1 mM methylsulfonyl fluoride, and incubated on ice for 10 min. After centrifugation at 14,000 g for 3 min (4 °C) the supernatant was carefully collected.

**RESULTS**

**Identification of the Minimal Protein Binding Sequence in the PTH mRNA 3′-UTR—**PT-cytoplasmic proteins specifically bind the full-length PTH mRNA transcript and a transcript for the PTH mRNA 3′-UTR. To identify the protein binding sequence in the PTH mRNA 3′-UTR we analyzed the binding of PT proteins to smaller RNA transcripts of the PTH mRNA 3′-UTR (Fig. 1A). Uniformly labeled transcripts were incubated with PT cytoplasmic extract and the mixture resolved on native polyacrylamide gels for REMSA. The free probe for all transcripts reacted with the major band. The molecular weight correlation of the REMSA structures of the RNA, because denaturing the RNA at 80 °C followed by slow renaturation at room temperature resulted in a single band on polyacrylamide gel. This renatured probe showed the same binding to PT proteins as the untreated transcript (not shown). The transcripts used for mapping the minimal protein binding element in the PTH 3′-UTR are shown.
in Fig. 1A. Transcripts of 174 and 38 nt that excluded the 60-terminal nt of the 3′-UTR did not bind proteins. A transcript of 234 nt consisting of the full-length UTR and transcripts of 100, 63, 50, and 40 nt (not shown) of the 3′-UTR showed a large protein-RNA complex on REMSA with PT proteins (Fig. 1B). This complex was reduced to a smaller complex after RNase T1 digestion of the bound protein RNA samples (Fig. 1B). Similar results were obtained when a transcript for the full-length PTH mRNA was analyzed (not shown). Transcripts of 30 (not shown) and 26 nt formed only the smaller protein-RNA complexes with or without treatment with RNase T1 (Fig. 1B) and the larger RNA-protein complex was not formed. These results show that a transcript of 40 nt was necessary for the formation of the larger protein-RNA complex that was obtained when the full-length PTH mRNA 3′-UTR transcript was analyzed. A 26-nt element was sufficient for protein binding and formed a complex that was similar to the complex formed with larger transcripts after treatment with RNase T1. Therefore, additional nucleotides in the 5′ of this element were necessary for formation of the larger complex that was formed in the absence of RNase T1. PT protein binding to these smaller transcripts (100, 63, and 40 nt) was increased by hypocalcemia and decreased by hypophosphatemia (Fig. 2), as with the full-length PTH transcript in our previous results (9). These results demonstrate that the regulation of cytosolic PT proteins to the PTH mRNA 3′-UTR increased by hypocalcemia and decreased by hypophosphatemia.

To further characterize the protein binding element in the PTH mRNA 3′-UTR we designed short single stranded antisense DNA oligonucleotides complimentary to portions of the 3′-UTR and analyzed their effect on protein-RNA binding by REMSA and UV cross-linking analysis. Fig. 4A shows the sequence of the 100 terminal nt of the PTH mRNA 3′-UTR including the 26 nt of the proposed protein-binding element and the antisense oligonucleotides used for the binding interference experiments. A representative REMSA for the binding of cytosolic PT proteins to the 3′-UTR transcript that had been preincubated with different antisense oligonucleotides is shown in Fig. 4B. The antisense oligonucleotides were annealed to the labeled 3′-UTR transcript that had been heated to 80 °C to unfold secondary structures in the RNA. PT protein extracts were then added and protein binding analyzed. Corresponding double-stranded DNAs were used as controls. Fig. 4B shows the 3′-UTR transcript after RNase T1 treatment and the protein-RNA complexes formed after addition of PT cytosolic extracts followed by RNase treatment. Preincubation of
Fig. 4. Antisense oligonucleotides corresponding to the protein-binding element prevent binding of PT proteins to the PTH mRNA 3'-UTR. A, the nt sequence corresponding to the terminal 100 nt of the 3'-UTR (as shown in Fig. 1A) and the single stranded antisense oligonucleotides used for binding interference are shown. The 26-nt protein-binding element is emphasized in **bold**. B, REMSA for the binding of PT proteins to the 3'-UTR without and with antisense oligonucleotides. All the samples were treated with RNase T1. Lane 1 shows the digested free probe in the absence of protein. Lane 2, shows the protein-RNA complexes formed in the presence of protein. For lanes 3–8, the RNA transcripts were preincubated at 80 °C with the different antisense oligonucleotides (1–6) depicted in Fig. 3A and then protein binding was analyzed by REMSA. Preincubation with the antisense oligonucleotides 3–5, which correspond to the protein-binding element or parts of it, prevented protein binding to the PTH mRNA 3'-UTR. C, UV cross-linking analysis for the binding of PT proteins to the 3'-UTR without and with antisense oligonucleotides. The assay was performed without (first lane) or after preincubation with antisense oligonucleotides 1–6, as for Fig. 3B either after unfolding at 80 °C (above) or without heating to 80 °C (below). Molecular mass markers (kDa) are shown on the right. Preincubation with the antisense oligonucleotides 3–5, which correspond to the protein-binding element or parts of it, prevented protein binding to the PTH mRNA 3'-UTR only if the RNA was denatured at 80 °C.
the PTH mRNA 3'-UTR transcript with antisense oligonucleotides 1, 2, and 6 (Fig. 4A) that did not span the protein binding sequences had no effect on protein binding (Fig. 4B). Preincubation with oligonucleotides spanning the 26-nt element or part of this sequence with or without 3'-flanking sequences (oligonucleotides 3, 4, and 5 in Fig. 4A) abolished the binding of PT proteins to the 3'-UTR (Fig. 4B). Corresponding sense or double-stranded DNAs had no effect on protein-RNA complex formation (not shown). The effect of antisense oligonucleotides on PT protein binding to the PTH RNA 3'-UTR was also analyzed by UV cross-linking experiments. In this assay RNA-binding proteins from cytosolic extracts are cross-linked to labeled transcript in solution and complexes resolved by denaturing SDS-PAGE. Fig. 4C shows that 3 cross-linked protein-RNA complexes of ∼110, 60, and 50 kDa were formed when a transcript for the PTH mRNA 3'-UTR was analyzed with PT protein extracts, as in our previous reports (9). When the transcript was denatured at 80 °C and preincubated with the antisense oligonucleotides the same inhibitory effect of the oligonucleotides corresponding to the binding region on protein binding was observed (Fig. 4C), similar to the REMSA (Fig. 4B). When the same UV cross-linking experiment was performed without denaturing the RNA by heating to 80 °C there was no effect of preincubation of the RNA with any of the oligonucleotides (Fig. 4C). In addition, when there was no preheating of the RNA, the relevant oligonucleotides did not interfere with binding also by REMSA (not shown). Together, these results indicate that the protein-binding recognition site of the 3'-UTR includes the element of 26 nt and that this region plays a role in protein-RNA binding. Moreover, the interference of binding only after unfolding the RNA at 80 °C suggests that protein binding to this region is dependent on secondary structures in the RNA.

Sequence analysis of the 26-nt element in the PTH mRNA revealed high conservation of the rat element in the PTH mRNA 3'-UTR to mouse (23 of 26 nt), human (19 of 26 nt), and canine (19 of 26 nt), with human and canine being identical (Table I). Such conservation of this sequence that lies outside of the coding region, among different species, suggests a functional role for this element.

**Functionality of the Protein-binding Region of the PTH mRNA 3'-UTR Inserted into Heterologous RNAs**—To demonstrate that the protein-binding region has a role in determining mRNA stability and response to calcium and phosphate, we inserted the fragment for the 63 nt of the PTH mRNA 3'-UTR, which contains the 26-nt element (Fig. 1A), into the structural gene of human growth hormone (GH). This is shown schematically in Fig. 5A. We used these constructs to study whether this inserted 63-nt sequence affected GH mRNA stability in an in vitro degradation assay with PT proteins of rats fed normal, low calcium, or low phosphate diets.

The in vitro degradation assay reflects the post-transcriptional regulation of PTH mRNA stability in vivo in response to changes in calcium and phosphate. A low calcium diet increases PTH mRNA levels in vivo and PT proteins from these rats stabilized the full-length PTH transcript using the in vitro degradation assay (Fig. 5B, upper panel, and Ref. 9). A low phosphate diet decreases PTH mRNA levels and led to rapid degradation of the PTH transcript by PT proteins from these rats (Fig. 5B, upper panel, and Ref. 9). We then analyzed the effect of the PTH mRNA 63-nt protein binding sequence on GH mRNA degradation in the presence of these PT proteins. With PT proteins from rats fed a normal diet, the chimeric GH transcript containing 63 nt of the PTH 3'-UTR was degraded more rapidly than the native GH transcript (t1/2 > 130 min: >180 min) (Fig. 5B, middle and bottom panels, normal diet). This suggests that this element is an instability element. We then studied the effect of PT proteins from hypocalcemic or hypophosphatemic rats on degradation of the GH transcripts. When the transcript for the native GH mRNA was analyzed with PT proteins from the different diets, there was no effect on native GH degradation (Fig. 5B, middle panel). In contrast, the chimeric GH transcript was stabilized by PT proteins from low calcium rats and more rapidly degraded with PT proteins of low phosphate rats (t1/2 > 180 min:30 min) (Fig. 5, B, bottom panel, and C), similar to the full-length PTH transcript (Fig. 5B, top panel). Therefore the protein binding sequences in the PTH mRNA 3'-UTR were sufficient to confer responsiveness to changes in PT proteins induced by dietary calcium and phosphate.

To determine the specificity of the effect of the protein-binding region, the protein-binding segment of 63 nt was inserted into a random sequence, the pCRII polylinker. In addition a shorter PTH mRNA 3'-UTR RNA of 38 nt, that itself did not bind PT proteins (Fig. 1A) was also inserted at the same site into the pCRII polylinker (Fig. 6A). The stability of the polylinker and chimeric RNAs was determined in the in vitro degradation assay with PT proteins. The PTH mRNA 63 nt was recognized and cleaved more rapidly by the PT extract (t1/2 = 10 ± 2 min, n = 3) than the RNA without the PTH mRNA insert (t1/2 = 35 ± 5 min, n = 3) (Fig. 6). Insertion of the shorter PTH RNA 35 nt did not destabilize the chimeric transcript (t1/2 = 40 ± 5 min, n = 3). These results suggest that the PTH 63-nt RNA destabilized the random RNA sequence of pCRII. This effect was similar to the effect of the 63 nt when it was inserted into a larger transcript, GH RNA, representing a cellular mRNA. The destabilizing effect was dependent on an intact protein-binding transcript, because a shorter transcript that disrupted protein binding did not have the same effect.

**DISCUSSION**

We have identified by gel shift binding, competition experiments, and the use of single-strand antisense oligonucleotides the minimal sequence of 26 nt for protein binding in the PTH mRNA 3'-UTR. Unlike gel shifts, UV cross-linking identified 40 nt as the smallest binding transcript and not the 26- or 30-nt transcripts. The 26 and 30 nt are part of the 40-nt binding transcript. It is not readily evident why the 26-nt element was not sufficient for protein binding by UV cross-linking. However, protein-RNA binding analysis by REMSA utilizes a native PAGE after incubating protein and RNA and therefore may be more physiological than denaturing gels of UV cross-linked RNA-protein complexes. Single-strand antisense oligonucleotides spanning the 26-nt core binding element, or parts of it, interfered with the binding of PT proteins to the 3'-UTR both by REMSA and UV cross-linking. The antisense data together with the competition experiments with the 26-nt element show that the 26-nt element is important for binding. It is possible that the larger sequence of 40 nt may represent the required sequence for protein-RNA interaction, with the 26 nt representing the core element.

We have previously shown that PT proteins from rats fed a low calcium diet, where there is a marked increase in PTH mRNA levels, have increased binding to the PTH mRNA full-length and 3'-UTR transcripts. In contrast, PT proteins from

| Table I: The 26-nt protein-binding element in the PTH mRNA 3'-UTR is conserved among species |
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| Rat | AATTATTTCTTCTTTTTAAAGTTATA |
| Murine | AATTGCCTTTTTAAAGTTAATA |
| Human | TATTGGTTATTCTTTTTAAAGTTAATG |
| Canine | TATTGGTTATTCTTTTTAAAGTTAATG |
rats fed a low phosphate diet where there is decrease in PTH mRNA levels, have decreased binding (9). We have now shown that protein binding to shorter transcripts (100, 63, and 40 nt) was also regulated by the different diets by UV cross-linking (Fig. 2). The binding of PT proteins to the 26-nt transcript by REMSA was similarly regulated by calcium and phosphate (not shown). These results demonstrate that the smaller transcripts retain the properties of the previously characterized full-length transcript (9).

The rat PTH mRNA 3'-UTR is 234 nt long. Sequence analysis of the PTH mRNA 3'-UTR of different species revealed a preservation of the 26-nt core protein-binding element in rat, murine, human, and canine 3'-UTRs. In particular, there is a stretch of 14 nt within the element that is present in all four species. In the 26-nt element, the identity among the species varies between 73 and 89%. For instance, the human and rat are 73% identical in the 26-nt element, and only 42% identical in their 3'-UTR (11). The canine and rat are 73% identical in their 26-nt element and 50% identical in their 3'-UTR (17). The human and canine are 100% identical in the 26 nt of the element and only 70% identical in their 3'-UTR. Comparison of the 26-nt sequence in rat and mouse showed 89% identity, however, their 3'-UTRs show a comparable degree of identity. All in all, this analysis suggests that the binding element may represent a functional unit that has been evolutionarily conserved, but sequencing of the 3'-UTR in many other species is needed to establish this conclusion.

One of the PT cytosolic proteins involved in the stabilization of the PTH transcript was identified by affinity chromatography to the PTH mRNA 3'-UTR as AUF1 (12). Gel shift assays showed that recombinant AUF1 bound the full-length, 3'-UTR (12) and shorter transcripts including the 26-nt core element. There is no PT cell line and therefore PTH mRNA stability was measured by an in vitro degradation assay with rat PT proteins (9). Recombinant AUF1 stabilized the PTH transcript in the in vitro degradation assay with rat PT proteins.

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**FIG. 5.** Insertion of the 63-nt protein-binding region of the PTH mRNA 3'-UTR into GH RNA conferred responsiveness to GH mRNA by PT proteins from rats fed low calcium or low phosphate diets in an in vitro degradation assay. A, schematic representation of the GH mRNA (above) and the chimeric GH mRNA containing the PTH 3'-UTR 63-nt element inserted at the end of the GH coding region (below). B, representative gels of in vitro degradation assays for labeled transcripts for PTH (top), GH (middle), and GH + 63 nt of the PTH 3'-UTR (bottom) with PT proteins from rats fed a normal, low calcium, or low phosphate diet. At timed intervals after protein and RNA incubation samples were removed for RNA analysis. C, time response curves of transcripts for GH + 63 nt of the PTH 3'-UTR after incubation with PT cytosolic proteins as in A (bottom panel). Each point represents the mean ± S.E. of three different experiments. The PTH 63-nt insert into the GH mRNA resulted in stabilization of the transcript with low calcium PT proteins and destabilization with low phosphate PT proteins, similar to the native PTH transcript. The PT proteins from the different diets did not affect the native GH transcript.

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**FIG. 6.** Insertion of the 63-nt protein-binding region of the PTH mRNA 3'-UTR into a random pCRII RNA resulted in decreased RNA stability that was dependent upon protein binding. A, cDNA fragments corresponding to 63 or 38 nt of the PTH mRNA 3'-UTR, as in Fig. 1A, were inserted into a polylinker of pCRII. Chimeric transcripts and a transcript of the polylinker were analyzed by in vitro degradation with PT proteins from normal rats. B, the last 3 lanes show the different transcripts at 60 min without added PT protein. The chimeric 63-nt transcript was less stable than the native and the chimeric 38-nt transcript.

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vitro degradation assay with PT proteins (12).

To study functionality of the protein-binding region, we used the 63-nt sequence to create a chimeric transcript. We inserted a 63-base pair fragment of the PTH 3'-UTR into the structural gene of human GH. The RNA transcribed from this chimeric gene includes the 26-nucleotide element within the 63 nt of the PTH-binding region. We then studied the effect of the protein-binding region to confer PT-like responsiveness to calcium and phosphate on a heterologous gene. Therefore, the effect of the 63 nt on RNA stability was dependent upon an intact protein-binding region both with the native GH transcript and therefore, to study the effect of the PTH 3'-UTR element on RNA decay, we performed in vitro degradation assays with cytosolic proteins from PT glands. With PT proteins, the chimeric transcript was less stable than the native GH transcript indicating that in this assay, with PT proteins the 63-nt element was an instability element. Furthermore, the chimeric GH mRNA transcript containing the 63 nt of the PTH mRNA responded to PT proteins from low calcium and phosphate similar to the PTH mRNA. Therefore, the 63-nt protein-binding region of the PTH mRNA 3'-UTR is both necessary and sufficient to regulate RNA stability and to confer responsiveness to changes in PT proteins by calcium and phosphate.

To document a correlation between protein-binding and RNA stability, the 63-nt protein-binding region or a shorter sequence of 38 nt that did not bind PT proteins were inserted into a random transcript of pCRII polylinker. The stability of these transcripts and the transcript of the original polylinker were then studied in the in vitro degradation assay with PT proteins from normal rats. The chimeric transcript was destabilized by the 63-nt insert and not by the truncated insert. This indicates that the 63-nt binding element also functions as an instability element in a random sequence. Moreover, the lack of effect of the truncated transcript indicates that a sequence, which binds PT proteins is necessary for the degradatory function in another RNA. A random transcript of a polylinker had been used by others to show that the ribonuclease cleavage site in the α-globin 3′-UTR could be conferred to a heterologous RNA (18). Therefore, the effect of the 63 nt on RNA stability was dependent upon an intact protein-binding region both with the native PTH RNA (9) as well as in the heterologous RNAs.

Increasing evidence demonstrates that mRNA decay is an actively regulated process that determines gene expression. This process involves trans-acting protein factors that interact with specific cis-elements in a mRNA and under different physiological conditions leads to rapid decay or stability. Defined elements in mRNAs bind specific RNA-binding proteins and have been shown to mediate in addition to RNA stability, subcellular localization and RNA translation (19, 20). The information encoded by such elements in the RNA can be packaged as primary sequences and secondary or tertiary structures or a combination of both. cis-Elements that determine mRNA stability or instability have been determined in a number of mRNAs. The primary sequence of some of these cis-acting elements is highly conserved among species (21).

defined example is the adenosine uridine-rich element. Repeats of this AUUUA pentamer in the 3′-UTR of mRNAs of various cytokines targeted them for rapid decay by their interaction with cytoplasmic trans-factors (22, 23). In the brains of Alzheimer’s patients there are increased levels of β-amyloid protein and often the amyloid precursor protein mRNA as well (21). A 29-base element in the 3′-UTR has been defined that is bound by trans-factors and determines the amyloid precursor protein mRNA decay (21). The iron response element is another well defined cis-element. This element in the ferritin 5′-UTR controls translation of this mRNA and in the transferrin receptor mRNA it is present in multiple iterations where it regulates mRNA stability (24, 25). We have now identified a novel cis-element in the PTH mRNA 3′-UTR that determines PTH mRNA stability in response to changes in serum calcium and phosphate.

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REFERENCES
1. Silver, J., and Kronenberg, H. M. (1996) in Principles of Bone Biology (Bilezikian, J. J., Raisz, L. G., and Rodan, G. A., eds) pp. 325–338 Academic Press, San Diego
2. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, J. (1995) Nature 376, 575–580
3. Naveh-Many, T., and Silver, J. (1999) J. Clin. Invest. 96, 1313–1319
4. Naveh-Many, T., Bahamimov, R., Liviu, N., and Silver, J. (1995) J. Clin. Invest. 96, 1786–1793
5. Kilav, R., Silver, J., and Naveh-Many, T. (1995) J. Clin. Invest. 96, 327–333
6. Slatopolsky, E., Finch, J., Denda, M., Ritter, C., Zhong, A., Dusso, A., MacDonald, P., and Brown, A. (1996) J. Clin. Invest. 97, 2534–2546
7. Almaden, Y., Canalejo, A., Hernandez, A., Ballesteros, E., Garcia-Navarro, S., Torres, A., and Rodriguez, M. (1996) J. Bone Miner. Res. 11, 970–976
8. Nielsen, P. K., Feldt-Rasmussen, U., and Olgaard, K. (1996) Nephrol. Dial. Transplant. 11, 1762–1768
9. Moallem, E., Silver, J., Kilav, R., and Naveh-Many, T. (1998) J. Biol. Chem. 273, 5253–5259
10. Hendy, G. N., Kronenberg, H. M., Potts, J. T. J., and Rich, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7365–7369
11. Kemper, B. (1986) CRC Crit. Rev. Biochem. 19, 353–379
12. Sela-Brown, A., Silver, J., Brewer, G., and Naveh-Many, T. (2000) J. Biol. Chem. 275, 7424–7429
13. Epstein, E., Sela-Brown, A., Ringel, I., Kilav, R., King, S. M., Benashski, S. E., Yisraeli, J. K., Silver, J., and Naveh-Many, T. (2000) J. Clin. Invest. 105, 505–512
14. Buzby, J. S., Brewer, G., and Nagent, D. J. (1999) J. Biol. Chem. 274, 33973–33978
15. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
16. Levy, S., Avni, D., Hariharan, N., Perry, R. P., and Meyuhas, O. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3319–3323
17. Rosed, T. J., Steinmeyer, C. L., McCausley, L. K., Grone, A., DeWille, J. W., and Brown, A. J. (1996) EMBO J. 15, 295–305
18. Wilhelm, J. E., and Vale, R. D. (1993) J. Cell Biol. 123, 269–274
19. Wilson, G. M., and Brewer, G. (1999) Prog. Nucleic Acids Res. Mol. Biol. 62, 257–291
20. Rajagopalan, L. E., and Malter, J. S. (1997) Prog. Nucleic Acids Res. Mol. Biol. 56, 257–286
21. Brewer, G. (1991) Mol. Cell. Biol. 11, 2460–2466
22. Loflin, P., Chen, C. Y., and Shyu, A. B. (1999) Gene Dev. 13, 1884–1897
23. Klausner, R. D., Rouault, T. R., and Harford, J. B. (1993) Cell 72, 19–28
24. Schlegl, J., Geggout, V., Schlager, B., Hentze, M. W., Westhof, E., Ehresmann, C., Ehresmann, B., and Romby, P. (1997) RNA 3, 1159–1172