Parathyroid hormone (PTH) gene expression is regulated post-transcriptionally by hypocalcemia and hypophosphatemia. This regulation is dependent upon binding of protective trans-acting factors to a specific element in the PTH mRNA 3'-untranslated region (UTR). We have previously demonstrated that a 63-nucleotide (nt) AU-rich PTH mRNA element is sufficient to confer regulation of RNA stability by calcium and phosphate in an \textit{in vitro} degradation assay (IVDA). The 63-nt element consists of a core 26-nt minimal binding sequence and flanking regions. We have now studied the functionality of this element in HER293 cells using reporter genes and showed that it destabilizes mRNAs for green fluorescent protein (GFP) and growth hormone, similar to its effect in the IVDA. To understand how the \textit{cis}-element functions as an instability element, we have analyzed its structure by RNase H, primer extension, and computer modeling. The results indicate that the PTH mRNA 3'-UTR and in particular the region of the \textit{cis}-element are dominated by significant open regions with little folded base pairing. Mutation analysis of the 26-nt core element demonstrated the importance of defined nucleotides for protein-RNA binding. In the GFP reporter system, the same mutations that prevented binding were also ineffective in destabilizing GFP mRNA in HER293 cells. This is the first study of an AU-rich element that relates function to structure. The PTH mRNA 3'-UTR cis-acting element is an open region that utilizes the distinct sequence pattern to determine RNA stability by its interaction with \textit{trans}-acting factors.

PTH\textsuperscript{1} has a central role in maintaining normal calcium and phosphate homeostasis as well as bone strength. The parathyroids (PTs) are regulated by calcium, phosphate, and 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} (1). Hypocalcemia dramatically increases PTH mRNA levels, secretion, and, after prolonged stimulation, PT cell proliferation (2). The increase in PTH mRNA levels is post-transcriptional. Phosphate also regulates PTH secretion, gene expression, and PT cell proliferation (3, 4). Dietary induced phosphate depletion dramatically decreases PTH mRNA levels, and this is also post-transcriptional (3). We have shown that this post-transcriptional regulation is mediated by protein-RNA interactions involving protein binding to a specific element in the PTH mRNA 3'-UTR that determines PTH mRNA stability (2, 5). Protein binding to the PTH mRNA 3'-UTR was increased by hypocalcemia and decreased by hypophosphatemia. The regulation of binding by calcium and phosphate was observed only in the PT and not in other tissues of the same rats. We have identified AUF1 (heterogeneous nuclear ribonucleoprotein D) as a protein \textit{trans}-acting factor that stabilizes the PTH mRNA (6). There is no PT cell line; therefore, we have utilized a cell-free mRNA \textit{in vitro} degradation assay (IVDA) to demonstrate the functionality of the parathyroid cytosolic proteins in determining the stability of the PTH transcript. This assay has been shown to authentically reproduce cellular decay processes. PT protein extracts from hypocalcemic rats stabilized and PT protein extracts from hypophosphatemic rats destabilized the full-length PTH transcript, correlating with mRNA levels \textit{in vivo}. Deletion of the protein-binding element of the PTH mRNA 3'-UTR resulted in stabilization of the transcript compared with the full-length PTH transcript. Moreover, calcium and phosphate did not regulate the stability of the truncated transcript. Therefore, the IVDA reproduces the \textit{in vivo} stabilizing effect of low calcium and destabilizing effects of low phosphate on PTH mRNA levels, and this regulation in the IVDA is dependent upon protein binding that protects an instability sequence in the PTH mRNA 3'-UTR (2). However, it is not known yet if the PTH mRNA 3'-UTR protein-binding element is the cleavage site itself or if it induces some exonuclease pathway that acts either on the 5’ or the 3’-end of the mRNA.

We have identified the minimal sequence for protein binding in the PTH mRNA 3'-UTR and determined its functionality. A minimal sequence of 26 nt in the PTH mRNA 3'-UTR was sufficient for protein binding. To study the functionality of the protein binding element in the context of a different RNA, a 63-bp fragment coding for the 26 nt and flanking nt was fused to growth hormone (GH) reporter gene. RNAs were transcribed \textit{in vitro}, and transcripts were subjected to IVDA with PT proteins. The chimeric GH PTH 63-nt transcript, as the full-length PTH transcript, was stabilized by PT proteins from rats fed a low calcium diet and destabilized by proteins of a low phosphate diet, correlating with PTH mRNA \textit{levels in vivo}. The native GH transcript was more stable than PTH and the chimeric RNAs and was not affected by PT proteins from the different diets. These results demonstrate that the protein binding region of the PTH mRNA 3'-UTR is both necessary and sufficient to confer responsiveness to calcium and phosphate and determines PTH mRNA stability and levels (5).
We have now studied the function of the PTH element in cells, using the heterologous cell line HEK293. Plasmids containing the native GH or GFP gene or chimeric genes containing the PTH 3′-UTR sequences at the 3′-end of the gene were transiently transfected into HEK293 cells, and mRNA levels were measured by Northern blot. There was a marked decrease in GH and GFP mRNA levels of the chimeric constructs compared with the WT constructs or chimeric constructs that contained a truncated sequence of the PTH mRNA element, and this effect was post-transcriptional. These results are in agreement with the decreased stability of the chimeric transcripts in the in vitro degradation assay with PT proteins, and they demonstrate the functional importance of the RNA protein binding region in the PTH 3′-UTR (5). In order to understand the mechanism by which the specific sequences in the PTH mRNA 3′-UTR regulate mRNA half-life both in vivo and in vitro and PTH gene expression in response to changes in calcium and phosphate, we studied the structure of the PTH mRNA 3′-UTR. Our results show that the 3′-UTR and in particular the 100 nt that include the 26-nt protein binding core and flanking sequences do not form a stable secondary structure. Mutation analysis confirmed the importance of specific sequence patterns for protein binding and for the destabilizing effect of the cis-acting element.

**Materials and Methods**

Construction of the chimeric GH and GFP mRNAs containing 63 and 100 nt, respectively, of the PTH mRNA 3′-UTR for transfection experiments. The 63- or 100-bp DNA corresponding to the PTH mRNA 3′-UTR 63- and 100-nt transcripts that were subcloned into PGEM-T vector (5) were excised. The 63-bp fragment or a 40-bp truncated fragment was inserted into the Smal site of the pS16-GH expression plasmid that contained the S16 ribosomal protein promoter linked to the GH structural gene lacking GH promoter sequences (7). The 63- or 40-bp fragments were inserted between the coding region and the 3′-UTR of GH mRNA. For GFP chimeric constructs, a 100-bp fragment of the PTH mRNA 3′-UTR consisting of the 63-bp element and flanking nucleotides in pCRII plasmid (Invitrogen) was inserted into the SacI-ApaI site of the MCS of pEGFP-C1 (Clontech) at the 3′-UTR for transfection experiments. The 63- or 100-bp DNA corresponding to the PTH mRNA element, the antisense oligonucleotide of the polylinker sequence of the pCRII plasmid that was also part of the GFP-PTH chimeric transcript was AAGTCCGAGCTCAGATCCTCGA.

**PTH RNA Structure by Primer Extension**—In vitro transcribed RNA for the full-length PTH mRNA, the 3′-UTR, or the 100 nt of the 3′-UTR (2 pmol) was partially digested by RNase T1 and U1 (Amersham Biosciences) and V1 (Pierce). RNase T1 digestion was performed at 37 °C for 10 min with 0.005–0.5 units of T1 in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 mM MgCl2, and 10 μg of tRNA. Digestion with U1 was performed at 55 °C for 10 min with 0.005–0.5 units in 33 mM sodium citrate (pH 5.5), 1.7 mM EDTA, and 3.5 mM urea. RNase V1 digestion was allowed for 30 min with 0.8–1.4 units in 25 mM Tris-HCl, 10 mM MgCl2, and 10 μg of tRNA. All digests were performed in a final volume of 10 μl. Reactions were stopped by phenol/chloroform extraction and precipitation with ethanol in the presence of 0.5 μl of NaCl and 1 μl of Quik-Precip (Edge Biosystems). A primer extension reaction was performed using a 5′-end-labeled antisense oligonucleotide complementary to the 3′-end of the PTH mRNA (antisense oligonucleotide 4 in Fig. 2A). An annealing mixture containing 2 pmol of 5′-end-labeled primer and 2 pmol of partially digested PTH RNA was incubated at 65 °C for 3 min and cooled at 4 °C for 15 min. Subsequently, the primer was extended toward the cleavage site by reverse transcription with 2 units of avian myeloblastosis virus reverse transcriptase (Promega) for 20 min at 37 °C. PCR products were run on 6% sequencing gels along with a sequencing reaction that was carried out with the same end-labeled primer. The sequencing reaction was performed using a Termo-sequenase kit (U.S. Biochemical Corp.). The gels were exposed to x-ray film.

**Mutations for Binding and Transfection Experiments**—The transcriptions of WT and mutated 26-bp element (Fig. 4A) were prepared from annealed sense and antisense oligonucleotides that were constructed to include the T3 RNA polymerase sequence (underlined). For the WT 26 nt, the sense oligonucleotide was AAAATACCTCCTACTAAAAAAGGAATTTTAAAGTATT and the antisense oligonucleotide was TAAATCTTTTTTTTTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT. The greater the thermodynamic stability and well ordered conformation of a local folded structure, the higher the Zscre and thus the lower free energy difference, Zscre.

The mutated PTH 100-nt sequence was prepared by PCR using mutated oligonucleotides for each construct as shown in Fig. 4 and then excised and inserted into the PTH expression plasmid as for the WT PTH 100-nt element.

**Computer Analysis**—The computer programs EDscan (9, 10) and Sig5th (11–13) were used to search for well ordered folding patterns containing the 63- or 100-bp DNA corresponding to the PTH mRNA 3′-UTR. The EDscan program computes a standard Z score, Zsocr, of the free energy difference, Eairat, between the lowest free energy structure of the local segment and its corresponding optimal restrained structure where all base pairs formed in the original minimal structure were prohibited. The greater the Zsocr of the segment is, the more well ordered the folded RNA structure is expected to be. Thus, the higher the Zsocr and Eairat, the more stable the thermodynamic stability and well-ordered conformation of a local folded segment. In practice, Eairat and Zsocr were computed by sliding a fixed length window in steps of one or a few nt from the 5′ to 3′ along the sequence.
The stability and statistical significance of a given RNA folding relative to others in the sequence were assessed by the two standard Z scores, significance score (SIGSCR) and stability score (STBSCR) (12, 13). The score SIGSCR of a local RNA segment was calculated by dividing the energy difference between the actual and mean random stabilities by the S.D. value of the random stabilities. The STBSCR was calculated by dividing the difference between the stability of the given segment and the mean stability of all overlapping segments by the S.D. value of the stabilities of these segments that were generated by taking successive, overlapping, fixed length segments stepped one or few nt at a time along the sequence. The primary approach SigStb was used to explore unusual folding regions whose SIGSCR and/or STBSCR are unusually small or large. The significant open region indicates that the structure folded by the biological segment is very unstable, and the structure of the random permutation of the biological sequences is statistically more stable than that of the natural sequence. The detailed instruction and online server of RNA2D (including EDscan and SigStb) are available on the World Wide Web at protein3d.ncifcrf.gov/shuyun/rna2d.html.

RESULTS

The Effect of the PTH mRNA 3′-UTR cis-acting element on the Expression of Reporter Genes in HEK293 Cells—We have previously identified a protein-binding cis-acting element in the PTH mRNA 3′-UTR. We demonstrated the function of a 63-nt element in an IVDA with parathyroid cytosolic proteins and the PTH mRNA transcript or a chimeric reporter gene transcript containing the PTH element (5). To show the functionality of the cis-element in vivo in cells, we studied its effect on reporter genes by transfection experiments. Since there is no PT cell line, we studied the function of the PTH binding element in the heterologous cell line HEK293. We used plasmids containing the native GH gene or the chimeric GH PTH 63- or 40-nt truncated element all driven by a S16 ribosomal protein promoter. The truncated 40-nt element that was shown not to bind PT proteins was used as a control (5). The 63- or 40-bp fragments were inserted into the same site of a GH expression plasmid, between the coding region and the 3′-UTR of GH mRNA. The plasmids were transiently transfected into HEK293 cells, and mRNA levels were measured at 24 and 48 h.

The stability and statistical significance of a given RNA folding relative to others in the sequence were assessed by the two standard Z scores, significance score (SIGSCR) and stability score (STBSCR) (12, 13). The score SIGSCR of a local RNA segment was calculated by dividing the energy difference between the actual and mean random stabilities by the S.D. value of the random stabilities. The STBSCR was calculated by dividing the difference between the stability of the given segment and the mean stability of all overlapping segments by the S.D. value of the stabilities of these segments that were generated by taking successive, overlapping, fixed length segments stepped one or few nt at a time along the sequence. The primary approach SigStb was used to explore unusual folding regions whose SIGSCR and/or STBSCR are unusually small or large. The significant open region indicates that the structure folded by the biological segment is very unstable, and the structure of the random permutation of the biological sequences is statistically more stable than that of the natural sequence. The detailed instruction and online server of RNA2D (including EDscan and SigStb) are available on the World Wide Web at protein3d.ncifcrf.gov/shuyun/rna2d.html.

The PTH mRNA 3′-UTR cis-acting element decreases GFP and GH reporter gene mRNA and protein levels. A, representative Northern blot for GH mRNA levels (top) and graph for the amount of GH protein secreted to the medium (bottom) of HEK293 cells that were transiently transfected with expression plasmids for WT GH (GH) or chimeric GH containing the PTH mRNA 3′-UTR 63-nt cis-acting element (GH+PTH63) or a truncated 40-nt element (GH+PTH40). The gels show four separate transfections for each expression plasmid. B, representative Northern blot (top) for GFP mRNA levels of HEK293 cells that were transiently transfected in quadruplicate, with expression plasmids for WT GFP or chimeric GFP containing the PTH mRNA 3′-UTR 100-nt cis-acting element (GFP+PTH100). The graph shows the expression of GFP protein by immunofluorescence. C, GFP mRNA levels are shown as percentages of L32 ribosomal mRNA levels in HEK 293 cells transiently transfected with the WT or chimeric GFP expression plasmids that were treated with actinomycin D (AD) or DRB for 16 h. The results of three independent experiments (four transfections for each treatment) are shown as percentages of mRNA levels in cells transfected with the WT GFP plasmid. Insertion of the mRNA 3′-UTR 63-nt cis-acting element into reporter mRNAs destabilized the mRNAs of both reporter genes.

The stability and statistical significance of a given RNA folding relative to others in the sequence were assessed by the two standard Z scores, significance score (SIGSCR) and stability score (STBSCR) (12, 13). The score SIGSCR of a local RNA segment was calculated by dividing the energy difference between the actual and mean random stabilities by the S.D. value of the random stabilities. The STBSCR was calculated by dividing the difference between the stability of the given segment and the mean stability of all overlapping segments by the S.D. value of the stabilities of these segments that were generated by taking successive, overlapping, fixed length segments stepped one or few nt at a time along the sequence. The primary approach SigStb was used to explore unusual folding regions whose SIGSCR and/or STBSCR are unusually small or large in the sequence by sliding a fixed length window along the sequence. The significant open region (14), whose SIGSCR and STBSCR are unusually large can be discovered. The term “significant open region” indicates that the structure folded by the biological segment is very unstable, and the structure of the random permutation of the biological sequences is statistically more stable than that of the natural sequence. The detailed instruction and online server of RNA2D (including EDscan and SigStb) are available on the World Wide Web at protein3d.ncifcrf.gov/shuyun/rna2d.html.

The PTH mRNA 3′-UTR ARE: An Unstructured Functional Element
40-nt construct compared with the WT GH construct at 24 h (not shown) or 48 h (Fig. 1A). Uniform transfection efficiency was demonstrated by cotransfection with a β-galactosidase expression plasmid. There was a similar decrease in secreted GH levels in the cell culture medium, shown as the mean for four transfections in each group (Fig. 1A). Because all GH constructs utilize the same S16 promoter, these results suggest that insertion of the 63-nt element decreased the stability of the GH transcript and not its transcription. These results are in agreement with the decreased stability of the GH chimeric transcript in the IVDA with PT proteins (5). These experiments demonstrate the functional importance of the RNA protein binding region in the PTH 3' UTR as an instability element not only in vitro in the IVDA but also in vivo in HEK293 cells. To show the functionality of the cis-element in the context of another RNA, we used the cDNA for bacterial GFP as a reporter gene. A fragment coding for the PTH 3'-UTR 100 nt that included the 63 nt and flanking nt was inserted into a cytomegalovirus promoter-driven GFP plasmid at the 3'-end of the GFP cDNA before the polyadenylation signal. Wild type and chimeric plasmids were transiently transfected in quadruplicate into HEK293 cells. After 24 and 48 h, the expression of GFP protein was visualized by immunofluorescence, and then total RNA was extracted, and GFP mRNA levels were measured by Northern blot. Insertion of the PTH element resulted in a marked decrease in GFP mRNA and protein levels in the chimeric gene compared with the WT gene in the four transfections in each group (Fig. 1B). As for the GH mRNAs, this result suggests that the PTH element affected GFP mRNA stability and not its transcription, since both GFP constructs utilized the same cytomegalovirus promoter. To confirm the post-transcriptional effect of the PTH instability element, actinomycin D or DRB were added to the GFP-transfected cells 24 h after transfection. WT and chimeric GFP mRNA levels were measured by Northern blot at 16 h after the addition of the transcription inhibitors to compare the decay of the two mRNAs (Fig. 1C). The basal levels of wild type GFP mRNA levels before the addition of the transcription inhibitors (control) were set as 100%, and L32 was used as a control mRNA. The results are expressed as percentage of GFP/L32 mRNA levels and are the mean of three independent experiments each comprising four repeat samples. The presence of the 100-nt element decreased basal GFP mRNA levels by 50% (Fig. 1C) as in Fig. 1B. After transcriptional inhibition by the addition of DRB or actinomycin D, GFP WT mRNA levels decreased to 70% of base line at 16 h. In contrast, the mRNA levels for GFP with the 100-nt PTH element were decreased to 5% of basal level (Fig. 1C). This demonstrates that the GFP mRNA with the 100-nt PTH element was significantly less stable than the WT GFP mRNA. Therefore, the PTH mRNA 3'-UTR 100-nt element is a destabilizing element, and its effect is post-transcriptional.

Secondary Structure of the PTH mRNA—To understand how the PTH mRNA 3'-UTR 63 and 100 nt that include the protein-binding element destabilize the PTH transcript as well as reporter RNA, we analyzed the putative structure of the element. We first used oligonucleotide-targeted RNase H digestion to study the domain structure of the PTH transcript. RNase H cleaves DNA/RNA hybrids, and we used antisense oligonucleotides spanning different regions of the PTH mRNA and RNase H digestion to study the secondary structure of the PTH transcript. Targeted sequences that are part of a stable double-stranded structure will not anneal to the oligonucleotide and will therefore not be cleaved by RNase H. In contrast, antisense oligonucleotides will anneal to open regions in the transcript and will therefore mediate cleavage by RNase H. In vitro transcribed PTH RNA representing the full-length PTH mRNA or endogenous PTH mRNA as part of total parathyroid RNA extracted from rat parathyroids was used. We targeted sequences along the PTH 3'-UTR and coding region, using 6–20-nt-long antisense oligonucleotides (Fig. 2A). The antisense oligonucleotides were incubated with the RNA, and then RNase H was added. After digestion, RNA was extracted and analyzed by Northern blots. Fig. 2 shows the results of representative gels of RNase H analysis using a full-length in vitro transcribed RNA (Fig. 2B) and PTH mRNA of total parathyroid RNA (Fig. 2C). The RNAs were incubated with oligonucleotide 1 in the sense orientation that does not anneal to the RNA or oligonucleotides 2–9 in the antisense orientation (Fig. 2A). As expected, the full-length in vitro transcribed PTH transcript without added oligonucleotides (Fig. 1B, first lane) or with oligonucleotide 1 in the sense orientation (Fig. 1B, lane 2) was not cleaved by RNase H. In addition, antisense oligonucleotides 7 and 9 did not mediate cleavage of the RNA, suggesting that they target sequences that are double-stranded. Oligonucleotide 8 showed partial cleavage, suggesting that the target of this oligonucleotide is only partly double-stranded. In contrast, the addition of antisense oligonucleotides 2–6 spanning the 100-nt element all led to complete cleavage of the PTH RNA. Cleavage by RNase H resulted in two fragments. The larger 5'.
The 100-nt element of the PTH mRNA decreased the stability of the GFP mRNA in transfection experiments in HEK293 cells (Fig. 1). If the open structure of the PTH mRNA 100-nt element is important for its function to regulate mRNA stability of PTH and reporter genes, we would expect that it remains as an unstructured region also in the GFP mRNA. We therefore studied the structure of the 100 nt of the PTH mRNA 3′-UTR in the GFP mRNA. In vitro transcribed chimeric GFP transcripts containing the PTH 100-nt element were analyzed by RNase H with antisense oligonucleotides 2, 3, 5, and 6 spanning the PTH 100-nt element (not shown). The antisense oligonucleotides for the PTH element mediated cleavage, but the sense oligonucleotide 1 and antisense oligonucleotide for the polylinker sequence of the pCRII plasmid that was also part of the chimeric transcript did not mediate cleavage (not shown). These results show that the same antisense oligonucleotides that mediated cleavage of the 100 nt in the full-length PTH transcript also mediated cleavage in the 100-nt PTH element within the chimeric GFP transcript. Therefore, the 100-nt element has an open structure in both PTH mRNA as well as when inserted into the GFP reporter gene. The open structure may be important for the function of this element.

RNase H can only give an estimation of the domain structure of the RNA because of the fact that RNase H cleaves RNA/DNA hybrids of 6–8 nt and cannot give information on the structure of specific nucleotides (15). To determine the structure of the PTH mRNA in greater detail, we performed primer extension experiments to identify the pairing of specific nt.

**Primer Extension Analysis of the PTH mRNA**—More detailed information regarding PTH mRNA structure was obtained by primer extension analysis of PTH transcripts that were partially cleaved by structure-specific RNases (8). Transcripts for the full-length PTH, the 3′-UTR, and the 100-nt functional region were partially cleaved by RNase T1 (specific for single-stranded guanine residues), RNase U2 (specific for single-stranded adenine residues), or RNase V1 (specific for double-stranded regions). To map the cleavage sites, the partially cleaved transcripts were subjected to primer extension with a 32P-end-labeled antisense primer to the 3′-end of the PTH transcript (oligonucleotide 4 in Fig. 2A). The resulting cDNA products were analyzed by sequencing gel electrophoresis. The results of a representative sequencing gel for the PTH mRNA 3′-UTR transcript are shown in Fig. 3. Without cleavage, primer extension of the in vitro transcribed PTH 3′-UTR resulted in a major band representing the full-length cDNA and additional laddering of smaller fragments. These frag-
mRNA 3' extension was performed with the full-length 700-nt PTH transcript. V00597, sequence alignment of the rat 26-nt element in mouse, human, dog, and cat PTH mRNA 3'-UTRs. The nt that are not conserved are shown in boldface type. The element was divided into two regions according to the similarity of the sequences. The first 10 nt are more variable than the stretch of 14 nt that are conserved in all species. B, the rat PTH mRNA 3'-UTR 26-nt element and mutations that were constructed to study their effect on protein-RNA binding and on the destabilizing function of the PTH mRNA 3'-UTR 26-nt region. Mutations 1–6 were created by replacing 2 or 3 nt at a time in the template DNA for the 26-nt transcript, as indicated. Shown on the right is a summary of the results of binding of PT protein extracts to RNAs that were transcribed from the mutated oligonucleotides as shown in Fig 5A. +, positive binding by REMSA; –, no binding. The accession numbers for the PTH sequences are as follows: rat, XO5721; mouse, AF066075; human, V06097; dog, U15662; cat, AF599667.

A. The Effect of Specific Mutation in the PTH cis-Element on Protein Binding and RNA Stability—Our results indicate that the PTH mRNA 3'-UTR does not have a stable secondary structure, at least in the absence of proteins. To study the role of specific nt in the cis-element in determining RNA stability, we constructed mutations in specific nucleotides of the cis-acting element and determined their effect upon binding and function. We have previously shown by binding experiments that the core 26-nt sequence in the 63-nt cis-acting element of the PTH mRNA 3'-UTR is sufficient for protein-RNA binding (5). The 26-nt element was also identified by sequence alignment of PTH mRNAs of different species (Fig. 4A). Comparison of the sequence of the 26-nt element of humans, rats, mice, dogs, and cats distinguishes between a variable region from nt 623 to 633 of the rat PTH mRNA and a highly conserved stretch of 14 nt from 634–648. We created mutations of specific nt in both regions of the 26-nt sequence and studied their effect on protein binding (Fig. 5A). We also studied their function as a destabilizing element within the 100-nt 3'-UTR element on GFP expression in transfected cells (Fig. 5B).

Fig. 4. Identification of the homologous sequences of the rat PTH mRNA 3'-UTR 26-nt protein-binding element in mice, humans, cats, and dogs. A, sequence alignment of the rat 26-nt element in mouse, human, dog, and cat PTH mRNA 3'-UTRs. The nt that are not conserved are shown in boldface type. The element was divided into two regions according to the similarity of the sequences. The first 10 nt are more variable than the stretch of 14 nt that are conserved in all species. B, the rat PTH mRNA 3'-UTR 26-nt element and mutations that were constructed to study their effect on protein-RNA binding and on the destabilizing function of the PTH mRNA 3'-UTR 26-nt region. Mutations 1–6 were created by replacing 2 or 3 nt at a time in the template DNA for the 26-nt transcript, as indicated. Shown on the right is a summary of the results of binding of PT protein extracts to RNAs that were transcribed from the mutated oligonucleotides as shown in Fig 5A. +, positive binding by REMSA; –, no binding. The accession numbers for the PTH sequences are as follows: rat, XO5721; mouse, AF066075; human, V06097; dog, U15662; cat, AF599667.
To study the effect of selected mutations on the functionality of the PTH mRNA 3′-UTR 100 nt, we mutated specific nt in the conserved region of the 26-nt element, within the PTH mRNA 3′-UTR 100 nt, and inserted the 100 nt into the expression plasmid for GFP as before. The plasmids were transiently transfected into HEK293 cells, four transfections in each group, and their effect on GFP mRNA levels was determined as in Fig. 1. The mutations used in each experiment are indicated in Fig. 5B. Insertion of the PTH mRNA 3′-UTR 100 nt WT sequence resulted in a decrease in GFP mRNA levels (Fig. 5) as before (Fig. 1B). Mutations in the conserved region of the PTH mRNA 3′-UTR 26-nt core binding element within the 100 nt abolished the destabilizing effect of the PTH mRNA 3′-UTR 100 nt (Fig. 5B, m1 and m2+3). This region was mutated in two discrete sets of nt of the PTH mRNA, and all of them lost their destabilizing function. The results shown are representative of three repeat experiments. In binding assays, mutating these nt resulted in loss of protein binding to these transcripts (Fig. 5A). These results suggest that the nt sequence preserved in the conserved region of the 26 nt is important both for binding and for destabilization of the PTH mRNA.

To test whether the PTH element is also in an open structure in vivo in the cell where it is bound to proteins and polyosomes, we used antisense oligonucleotides to the 26-nt element. We cotransfected the chimeric GH reporter gene containing the 63- or 40-nt truncated element of the PTH mRNA 3′-UTR with a specific antisense oligonucleotide or sense oligonucleotide as control. The antisense oligonucleotide decreased GH mRNA levels compared with the sense transfected cells when the chimeric gene contained the full 63-nt element (Fig. 6). The truncated GH PTH 40-nt element mRNA levels were not affected by either oligonucleotide (Fig. 6).

These results suggest that the antisense oligonucleotide anneals to the sequence in the 63-nt element of the chimeric transcript and thereby induces endogenous RNase H cleavage of the RNA-DNA hybrid (20). This effect is specific for the antisense, because the sense oligonucleotide had no effect, and a truncated 40-nt element in the GH mRNA was not a target to either oligonucleotide. These results support our structure analysis in vitro that the PTH mRNA 3′-UTR cis-acting element is an open structure.

DISCUSSION

We have developed a reporter assay for the function of the PTH mRNA 3′-UTR defined element. Using two reporter genes, GFP and GH, we have shown that the protein-binding element in the PTH mRNA 3′-UTR destabilizes the reporter mRNAs in HEK293 cells. This is in agreement with our results using an in vitro degradation assay of chimeric GH PTH mRNA 3′-UTR 63-nt and parathyroid cytosolic proteins. In the IVDA the PTH mRNA, 3′-UTR 63 nt destabilized the GH transcript and conferred responsiveness to parathyroid extracts from rats fed low calcium or low phosphate diets. The low calcium PT proteins stabilized and the low phosphate PT proteins destabilized the chimeric transcript compared with control, similar to the full-length PTH transcript (5). There is no PT cell line, and there is no other cell line that responds to changes in extracellular calcium and phosphate like the parathyroid does. However, our results show that HEK293 cells are able to reproduce the destabilizing effect of the PTH mRNA 3′-UTR element. RNA stability reflects the balance between stabilizing and destabilizing cytosolic factors that act on the transcribed mRNA. The PTH mRNA 3′-UTR-binding proteins that protect the RNA from degradation in the PT cytosol are ubiquitous, but only in the PT is their binding and function regulated by calcium and phosphate. In the HEK293 cell, there is a given amount of cytosolic binding protein that after transfection binds and prevents degradation mediated by the 100-nt destabilizing element. Excess chimeric GFP PTH mRNA 3′-UTR 100 nt saturates the protective binding proteins and results in the decrease in chimeric GFP mRNA levels but not of the native GFP that is not affected by these factors.

We used the GFP-PTH mRNA 3′-UTR 100-nt chimeric gene to determine the role of specific nt in destabilizing the reporter gene. We have previously shown that there is a conserved 26-nt core element in the PTH mRNA 3′-UTR that is sufficient for protein binding. This minimal binding element is conserved among humans, dogs, cats, rats, and mice compared with the
rest of the 3'-UTRs that are far less conserved. We mutated specific nt in the PTH mRNA 3'-UTR 26-nt core protein-binding element. We studied the effect of these mutations on binding of PT proteins to the mutated PTH transcript and their destabilizing function in the chimeric GFP gene. The mutated transcripts of the 26 nt that did not bind PT proteins in REMSA also did not destabilize the GFP mRNA in the transfected HEK293 cells. All of the mutations that disrupted the function and binding of the PTH mRNA 3'-UTR element were at the 3'-region of the 26-nt element and localized to 14 nt. These 14 nt were completely preserved within mouse, human, dog, and cat PTH mRNA 3'-UTR, suggesting that this sequence is of functional importance. The 5'-portion of the 26-nt core element is not as conserved among the species. Mutations in this less conserved region did not affect binding, suggesting that the sequence of this region may be of less functional significance. In addition, a transcript for the human 26-nt element bound human and rat parathyroid proteins with the same affinity as the rat element, indicating that the 5' nt that differ in the variable region of the human PTH mRNA 26-nt element do not affect the binding of PT rat proteins.

mRNA stability and its translation may be determined by both sequence and structure of the RNA. A well defined example is the regulation by iron of ferritin translation and the transferrin receptor mRNA stability by a stem and loop iron-responsive element (21, 22). A stem loop structure is also involved in determining the stability of histone H4 mRNA. Interaction of this stem loop structure, located at the 3'-end of the mRNA with cellular proteins promotes degradation of the mRNA (23). The most widely spread family of instability sequences are the AREs that generally promote rapid degradation of the mRNA (24). The most widely spread family of instability sequences is the AREs that generally promote rapid degradation of ARE containing mRNAs. The most widespread family of instability sequences is the AREs that generally promote rapid degradation of ARE-containing mRNAs. The most widespread family of instability sequences is the AREs that generally promote rapid degradation of ARE-containing mRNAs.

Computer analysis as well as the results of the primer extension and RNase H analysis showed that the 3'-region of the PTH mRNA 3'-UTR has no stable structure. Mutation analysis also supports the importance of a sequence rather than structure. This suggests that this RNA region is open, which is an unusual finding. The PTH mRNA 3'-UTR functional region is an AU-rich element that binds AUFI, which stabilizes the PTH transcript (6). However, it does not have the canonical AUUUA pentamer, which therefore classifies it as a type III ARE (24). The structure of the mRNAs that contain AREs has not yet been determined. It would be of interest to know whether there is a contribution of structure to the function of any of the other AREs in particular type III AREs. Wilson et al. (27) showed that magnesium-induced conformational changes in the ARE of tumor necrosis factor-α mRNA inhibited AUFI binding. In addition, using resonance energy transfer to characterize structural changes in RNA substrates in response to cations and AU binding, it was shown that association of AUFI with an ARE may function to remodel local RNA structures that may be important for its function (18). The results presented here with the PTH mRNA 3'-UTR, showing that it is dominated by significant open regions, are the first to indicate that the ARE functions in regulating mRNA stability without having a defined structure. It is possible that in the presence of cellular RNA-binding proteins, the unstable structure of the free mRNA may adopt a more defined structure. We showed that an antisense oligonucleotide to the PTH mRNA 3'-UTR 26-nt element specifically decreased mRNA levels in transfected HEK293 cells. This is most likely due to annealing that induces degradation of the RNA by endogenous RNase H (20). The inference is that the target RNA is in a configuration that allows annealing of the antisense oligonucleotide target. For this to happen, the target RNA is presumably open in vivo in the cell as it is in vitro. In any case, most of the assays for RNA regulation, such as protein-RNA binding, IVDA, in vitro translation, etc., utilize in vitro transcribed RNA. The fact that these assays reproduce the in vitro regulation of mRNA fate suggests that the structure that may be induced by the endogenous proteins is not essential for these in vivo processes that can be simulated in vitro. In particular, the regulation of PTH mRNA binding and RNA stability by calcium and phosphate can be demonstrated with in vitro transcribed PTH RNA, suggesting that this regulation occurs with an open structured PTH mRNA 3'-UTR.

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REFERENCES
1. Silver, J., Naveh-Manly, T., and Kronenberg, H. M. (2002) in Principles of Bone Biology (Bilenizen, J. B., Raisz, L. G., and Rodan, G. A., eds) pp. 407–420, Academic Press, Inc., San Diego
2. Moallem, E., Silver, J., Kilav, R., and Naveh-Manly, T. (1998) J. Biol. Chem. 273, 5252–5259
3. Kilav, R., Silver, J., and Naveh-Manly, T. (1995) J. Clin. Invest. 96, 327–333
4. Naveh-Manly, T., Bahashinov, R., Livni, N., and Silver, J. (1995) J. Biol. Chem. 270, 1786–1793
5. Kilav, R., Silver, J., and Naveh-Manly, T. (2001) J. Biol. Chem. 276, 8724–8733
6. Sela-Brown, A., Silver, J., Brewer, G., and Naveh-Manly, T. (2000) J. Biol. Chem. 275, 7424–7429
7. Levy, S., Avni, D., Harsharan, N., Perry, R. P., and Meyuhas, O. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3319–3323
8. Argaman, L., and Altuvia, S. (2000) J. Mol. Biol. 300, 1101–1112
9. Le, S. Y., Chen, J. H., Konings, D., and Maizel, J. V. (2002) Proceedings of the 2002 International Conference on Mathematics and Engineering Techniques in Medicine and Biological Sciences, pp. 41–45, CSREA Press, Las Vegas, NV
10. Le, S. Y., Chen, J. H., Konings, D., and Maizel, J. V. (2003) Bioinformatics 19, 354–361
11. Le, S. Y., Chen, J. H., Currey, K. M., and Maizel, J. V. Jr. (1988) Comput. Appl. Biochem. 4, 153–159
12. Le, S. Y., and Maizel, J. V. Jr. (1989) J. Theor. Biol. 138, 495–510
13. Le, S. Y., Chen, J. H., and Maizel, J. V. (1990) in Structure and Methods VI: Human Genome Initiative and DNA Recombination (Sarma, R. H., and Sarma, M. H., eds) Adenine Press, Schenectady, NY
14. Le, S. Y., Chen, J. H., Chatterjee, D., and Maizel, J. V. (1989) Nucleic Acids Res. 17, 3275–3288
15. Donis-Keller, H. (1979) Nucleic Acids Res. 7, 179–192
16. Macdonald, L. E., Durbin, R. K., Dunn, J. D., and McAllister, W. T. (1994) J. Mol. Biol. 238, 145–158
17. He, B., Kukarin, A., Temiakov, D., Chin-Bow, S. T., Lyakhov, D. L., Rong, M., Durbin, R. K., and McAllister, W. T. (1998) J. Biol. Chem. 273, 18802–18811
18. Wilson, G. M., Sutphen, K., Moutaifis, M., Sinha, S., and Brewer, G. (2001) J. Biol. Chem. 276, 38409–38419
19. Mead, D. A., Szeresova-Skupova, E., and Kemper, B. (1986) Protein Eng. 1, 67–74
20. Toulme, J. J. (2001) Nat. Biotechnol. 19, 17–18
21. Klauser, R. D., Roselli, T. P., and Harford, J. B. (1993) Cell 72, 19–28
22. Theil, E. C., and Eisenstein, R. S. (2000) J. Biol. Chem. 275, 40659–40662
23. Ross, J. (1995) Microbiol. Rev. 59, 423–450
24. Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Raijmakers, R., Pruijn, G. J., Stoecklin, G., Moreci, M., Mann, M., and Karin, M. (2001) Cell 107, 451–464
25. Wilson, G. M., Sutphen, K., Chuang, K., and Brewer, G. (2001) J. Biol. Chem. 276, 8695–8704
26. R. Kilav, O. Bell, S.-Y. Le, J. Silver, and T. Naveh-Manly, unpublished observations.