In Vitro Evidence that Upstream of N-ras Participates in the Regulation of Parathyroid Hormone Messenger Ribonucleic Acid Stability

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Calcium and phosphate regulate PTH gene expression posttranscriptionally through the binding of trans-acting factors to a defined cis-acting instability element in the PTH mRNA 3′-untranslated region (UTR). We have previously defined AU-rich binding factor 1 as a PTH mRNA binding and stabilizing protein. We have now identified, by affinity chromatography, Upstream of N-ras (Unr) as another PTH mRNA 3′-UTR binding protein. Recombinant Unr bound the PTH 3′-UTR transcript, and supershift experiments with antibodies to Unr showed that Unr is part of the parathyroid RNA binding complex. Finally, because there is no parathyroid cell line, the functionality of Unr in regulating PTH mRNA levels was demonstrated in cotransfection experiments in heterologous human embryonic kidney 293 cells. Depilation of Unr by small interfering RNA decreased simian virus 40-driven PTH gene expression in human embryonic kidney 293 cells transiently cotransfected with the human PTH gene. Overexpression of Unr increased the rat full-length PTH mRNA levels but not a PTH mRNA lacking the terminal 60-nucleotide cis-acting protein binding region. Unr also stabilized a chimeric GH reporter mRNA that contained the rat PTH 63-nucleotide cis-acting element but not a truncated PTH element. Therefore, Unr binds to the PTH cis element and increases PTH mRNA levels, as does AU-rich binding factor 1. Our results suggest that Unr, together with the other proteins in the RNA binding complex, determines PTH mRNA stability. (Molecular Endocrinology 20: 1652–1660, 2006)

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Abbreviations: AU1, AU-rich binding factor 1; CSD, cold shock domain; 2-D, two-dimensional; DTT, dithiothreitol; GFP, green fluorescent protein; h, human; HEK, human embryonic kidney; mCRD, major coding region determinant; nt, nucleotide; PT, parathyroid; r, recombinant; REMSA, RNA EMSA; siRNA, small interfering RNA; Unr, Upstream of N-ras; UTR, untranslated region.

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now show that Unr regulates PTH mRNA gene expression. Knock-down of Unr by small interfering RNA (siRNA) led to a decrease in the expression of the human PTH gene, but not a truncated PTH gene, in cotransfection experiments in human embryonic kidney (HEK)293 cells. In these cells, overexpression of Unr stabilized the rat PTH mRNA and a chimeric reporter mRNA containing the rat 63-nt PTH mRNA 3' UTR regulatory element, but not a PTH mRNA lacking the terminal 60 nt nor a reporter mRNA containing a 40-nt truncated PTH element. Our results indicate that Unr is a functional component of the PTH mRNA stabilizing complex and may contribute to the regulation of PTH mRNA stability.

RESULTS

Identification of Unr as a PTH mRNA 3'-UTR Binding Protein

Affinity purification was performed as previously described (3) using rat brain cytosolic extracts enriched for RNA binding proteins by a heparin Sepharose column. The extracts were chromatographed on a PTH RNA affinity column. The column consisted of transcripts for the PTH mRNA 3'-UTR linked to cyanogen bromidine-activated Sepharose. The proteins that bound the column were eluted by increasing NaCl concentrations, and analyzed by UV cross-linking to the PTH 3'-UTR probe (data not shown). The proteins that were eluted between 220 and 500 mM exhibited maximum binding activity. The corresponding fractions were then combined, concentrated, and analyzed by two-dimensional (2-D) gel electrophoresis. Two gels were run in parallel. An aliquot of the combined positive fractions was run on a 2-D gel and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau red for protein detection and then incubated with a reporter mRNA containing the rat 63-nt PTH mRNA 3'-UTR regulatory element, but not a PTH mRNA lacking the terminal 60 nt nor a reporter mRNA containing a 40-nt truncated PTH element. Our results indicate that Unr is a functional component of the PTH mRNA stabilizing complex and may contribute to the regulation of PTH mRNA stability.

Unr Specifically Binds the PTH mRNA 3'-UTR and Is Part of the PT Protein-RNA Complex

Purified recombinant Unr (rUnr) protein was tested for interaction with PTH mRNA 3'-UTR by RNA EMSA (REMSA). rUnr showed a dose-dependent specific RNA binding (Fig. 2A). In some experiments, a smaller complex present in lower intensity was also present that was also shifted further with an increase in rUNR concentration (Fig. 2A). Excess unlabeled transcript for the PTH mRNA 3'-UTR competed for binding already at 10-fold excess (Fig. 2A, lanes 8 and 9). A transcript for PTH mRNA 3'-UTR without the terminal 60-nt cis-acting element of the 3'-UTR was used as a nonspecific competitor and neither bound (data not shown) nor competed (Fig. 2A, lane 7). To show that Unr is part of the PTH mRNA 3'-UTR protein binding complex in the PT gland, we added Unr antibody to rat PT protein extracts and then analyzed the binding to PTH mRNA 3'-UTR by REMSA. The complex formed by PT cytosolic proteins and the PTH mRNA 3'-UTR transcript was shifted after pretreatment of the extracts with increasing concentrations of the Unr antibody (Fig. 2B). Preimmune serum had no effect on complex size (Fig. 2B, lane 7). These results show that Unr binds specifically to the PTH mRNA 3'-UTR and that it is part of the protein-PTH mRNA 3'-UTR in a PT extract.

Depletion of Unr by siRNA Decreases PTH Gene Expression in Transfected HEK293 Cells

We have shown previously that protein binding to the PTH mRNA 3'-UTR correlates with increased PTH

Fig. 1. Identification of the PTH RNA 3'-UTR Binding Proteins by Northwestern Analysis

An aliquot of rat brain proteins from the combined positive fractions eluted from the rat PTH mRNA 3'-UTR affinity column were run on a 2-D gel and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau red (left) and then incubated with 32P-labeled rat PTH 3'-UTR transcript for Northwestern analysis (right). Several prominent spots were identified, including a set of proteins of approximately 50 kDa, that are the different isoforms of AUF1 and two single proteins of approximately 60 and approximately 100 kDa. The larger protein was identified by microsequencing as Unr. Unr and AUF1 are shown on the right, and the molecular weight markers are shown on the left.
with the control oligonucleotide (Fig. 3A). The depletion of Unr led to a decrease in hPTH mRNA and secreted PTH (Fig. 3B and C). The decrease in PTH gene expression correlated with the amount of siRNA added and the resultant Unr protein remaining in the reaction. There was no effect on the expression of the truncated PTH gene as measured by PTH secreted into the medium (Fig. 3C). The truncated PTH mRNA lacks the terminal 60-nt protein-binding region that we have shown previously is necessary for the regulation of PTH mRNA stability by calcium and phosphate (1, 11). These results demonstrated that Unr is critical to PTH gene expression and is dependent on the PTH mRNA 3′-UTR protein binding site. The cotransfections of siRNAs targeted to both Unr and AUF1 and the hPTH expression plasmid did not have an additive effect (data not shown). Cotransfection with green fluorescent protein (GFP) expression plasmid showed transfection efficiency of more than 90% in all experiments, as measured by fluorescent microscopy (data not shown).

Overexpression of Unr Increases the Expression of the PTH Gene and a Reporter GH Gene Containing the Rat PTH mRNA 3′-UTR 63-nt cis-Acting Element

The effect of overexpression of Unr on the expression of the full-length and truncated rat PTH mRNA was studied in cotransfection experiments in HEK293 cells. The basal expression of the truncated PTH plasmid showed a more than 4-fold increase compared with the full-length PTH cDNA containing plasmid, confirming its role as an instability element also in this assay (1). Cotransfection with an expression plasmid for Unr-FLAG and a plasmid for the full-length PTH cDNA led to a more than 2-fold increase of PTH mRNA levels in HEK293 cells compared with transfection with a plasmid that did not contain the Unr cDNA (Fig. 4A). However, Unr did not increase PTH mRNA levels using a plasmid lacking the terminal 60 bp of the PTH cDNA that disrupted the protein-binding element (Fig. 4A). Surprisingly, in some experiments, there was in fact a moderate unexplained decrease in truncated PTH mRNA levels by Unr (Fig. 4A). Both PTH constructs were driven by the same cytomegalovirus promoter and therefore the effect of Unr to increase the levels of the full-length PTH mRNA was not due to an effect on the common promoter. These results demonstrate that Unr stabilizes the PTH mRNA and that this is dependent on the presence of an intact protein binding cis-acting instability element. To study whether the 63-nt element is sufficient for the stabilizing effect of Unr, we expressed Unr in HEK293 cells together with a GH reporter gene containing either a 63-bp insert of the rat PTH mRNA 3′-UTR instability binding region or a truncated nonfunctional 40-bp insert. The 63-nt intact PTH mRNA instability element decreased GH mRNA levels compared with the truncated element as before (Fig. 4, B and C) (8). Cotransfection with an
expression plasmid for Unr FLAG led to a 3-fold increase in GH-PTH 63-nt mRNA levels but not in the GH-PTH 40-nt truncated mRNA (Fig. 4, B and C). Western blot using the FLAG antibody confirmed the expression of Unr FLAG in the transfected cells (Fig. 4D). Cotransfection with GFP expression plasmid was used to confirm transfection efficiency of more than 90% in the experiments, as measured by fluorescent microscopy (data not shown). These results indicate that the PTH mRNA 3′-UTR 63-nt cis element is both necessary and sufficient for the effect of overexpressed Unr to increase PTH mRNA levels. Together with the siRNA experiments, they underline the importance of Unr in the regulation of PTH gene expression.

**DISCUSSION**

The regulation of PTH mRNA stability is mediated by the binding of PT cytosolic proteins to an instability...
element in the PTH mRNA 3′-UTR (1, 7). Increased binding of PT cytosolic proteins to this element is associated with stabilization of the PTH RNA in in vitro degradation assays and with elevated PTH mRNA levels in vivo in hypocalcemic rats. In hypophosphatemic rats, there is decreased binding, RNA stability, and PTH mRNA levels. This cis-acting element leads to destabilization of reporter GH and GFP mRNAs in HEK293 cells and in in vitro degradation assays with PT cytosolic extracts (Fig. 4) (8). AUF1 is part of the PTH mRNA binding complex and stabilizes the PTH mRNA (3, 12). We have previously shown that AUF1

![Figure 4](https://academic.oup.com/mend/article-abstract/20/7/1652/2738406/Unr-on-PTH-mRNA-Stability)

**Fig. 4.** Overexpression of Unr Increases Rat PTH mRNA and Chimeric GH-Rat PTH 63-nt Element mRNA Levels But Not a Truncated PTH mRNA or a GH mRNA Containing a PTH mRNA 40-nt Truncated Element in Transfection Experiment in HEK293 Cells

A, Northern blot for rat PTH mRNA in HEK293 cells that were transiently cotransfected in triplicate with an expression plasmid for a truncated PTH mRNA lacking the terminal 60-nt cis-acting element or for the full-length PTH mRNA and an expression plasmid for Unr-FLAG or an empty vector (PSG). Lower panel shows hybridization to L32 ribosomal protein mRNA. Quantification of the radiograms is shown below the gels as PTHmRNA/L32 mRNA expressed as percentage of truncated PTH mRNA levels without Unr-FLAG expression. B, Northern blot for GH mRNA in HEK293 cells that were transiently cotransfected in triplicate with an expression plasmid for GH mRNA containing either the rat PTH mRNA 3′-UTR 63-nt cis element or a truncated PTH 40-nt element and an expression plasmid for Unr-FLAG or a control plasmid (PSG). The bottom panel shows rehybridization for L32 mRNA. C, Quantification of the radiograms in B presented as PTHmRNA/L32 mRNA (percentage of the levels of GH-PTH 40 without Unr-FLAG). *, P < 0.05 compared with levels of GH-PTH 40 without Unr-FLAG. Similar results were obtained in three repeat experiments. D, Western blot of cytosolic extracts from cells in B transfected with GH-PTH63 without and with Unr-FLAG, using antibodies to the FLAG tag of Unr. Unr-FLAG was similarly expressed in cotransfection experiments with the other PTH and GH expression plasmids.
levels do not change in the PTs of rats fed control, low-calcium, or low-phosphorus diets, despite large differences in PTH mRNA levels and protein-RNA binding. However, there were significant differences in posttranslational modifications of AUF1 as seen by 2-D gels (12). We have now identified Unr as one of the proteins in the PTH mRNA 3'-UTR protein complex. In addition, we show that UNR increases PTH mRNA levels in a heterologous cell line because there is no PT cell line.

The function of Unr was demonstrated in HEK293 cells by depletion experiments using siRNAs targeted to the Unr mRNA. Cotransfection with siRNA oligonucleotides complementary to the Unr mRNA together with hPTH gene expression plasmid driven by a viral promoter led to a specific decrease in PTH mRNA levels and secreted PTH. We have previously shown that depletion of AUF1 in these cells led to a similar effect on hPTH gene expression and secretion (12). However, when siRNAs to both Unr and AUF1 were added, there was no additive effect (data not shown). This could be because depletion of either protein alone maximally suppressed the expression of PTH in this system. This would suggest that AUF1 and Unr can act independently to regulate PTH mRNA levels, which is consistent with the ability of overexpression of Unr alone to increase PTH mRNA levels. Alternatively, if the two proteins act as a heterodimer, the depletion of either protein would have the same effect as when both are depleted. It should be noted that knock-down experiments are often more meaningful than overexpression experiments, because overexpression may lead to oversaturation and also because the overexpressed protein may not necessarily undergo the specific posttranslational modifications of the native protein. We do not know whether endogenous Unr is regulated posttranslationally in vivo in the PT by calcium and phosphate, because the antibody for rat Unr was not sensitive enough for the analysis of Unr.

The PTH mRNA 3'-UTR contains a 26-nt core element that is also present in the human, feline, and canine species (11). We have shown that the region that contains the 26-nt element in the human and rat 3'-UTRs confers instability to reporter genes in HEK293 cells (11), similar to the effect of the rat PTH mRNA 3'-UTR sequences (7). To study the role of overexpression of Unr on the PTH mRNA 3'-UTR cis element, we used the rat PTH mRNA where this element has been defined in detail (7, 8). When overexpressed in HEK293 cells, Unr led to an increase in PTH mRNA levels in cotransfection experiments using the full-length PTH cDNA but not a truncated PTH cDNA lacking the terminal 60 bp that includes the 26 bp coding for the cis element. A similar effect of Unr expression was found using a chimeric GH reporter transcript containing the rat PTH mRNA 3'-UTR 63-nt cis-acting instability region that includes the 26-nt element. This effect of Unr was not seen with a control GH PTH mRNA 3'-UTR 40-nt truncated element. Therefore, Unr is part of the PTH mRNA 3'-UTR binding complex and acts to stabilize the PTH mRNA in HEK293 cells through its interaction with the 3'-UTR cis-acting element.

The unr gene was identified as a transcription unit located immediately upstream of N-ras in the genome of several mammalian species (13, 14). It encodes a 85-kDa protein highly conserved in mammals, which has a broad tissue and cell type distribution, and is largely cytoplasmic (15, 16). The Unr protein which is composed of five cold shock domains (CSD) is a unique member of the superfamily of CSD proteins (17). The domain of approximately 70 amino acid residues mediates binding to single-stranded DNA and RNA. The eukaryotic CSD proteins Y-box factors have been implicated in a variety of processes, including the regulation of splicing, RNA turnover and translation (18–21).

Unr has been shown to regulate mRNA translation. It has been characterized as a factor required for internal initiation of viral (rhinovirus and poliovirus) (22) and cellular (Apaf-1) RNAs (22, 23). Unr is thought to act as a RNA chaperone by changing the structure of the internal ribosome entry site to permit initiation of rhinovirus and Apaf-1 translation (23, 24). In addition, Unr is central to the translationally coupled mRNA turnover mediated by the major coding region determinant (mCRD) in c-fos mRNA (9) (10). The mechanisms by which Unr stimulates internal initiation of translation and mRNA turnover processes differ. In c-fos mRNA, Unr is part of a complex of five RNA-binding proteins that bind to a 24-nt instability element. This complex then induces destabilization of c-fos mRNA in a translation-dependent manner (9). Grosset et al. (9) showed by coimmunoprecipitation experiments that AUF1 (hnRNP D) interacts in NIH 3T3 cells with Unr; poly(A) binding protein; PAIP-1, a poly(A) binding protein interacting protein; and NSAP1, an hnRNP R-like protein. Unr both binds the mCRD and interacts directly with poly(A) binding protein. Chang et al. (10) identified a human poly(A) nuclelease CCR4 that also associates with Unr. They proposed a model in which the mCRD/Unr complex serves as a “landing/assembly” platform for formation of a deadenylation/decay mRNA-protein complex on a mCRD-containing transcript (10). In the present study, Unr is shown to be part of the PTH mRNA stabilizing complex as is AUF1. The effect of Unr to stabilize the PTH mRNA is in contrast to the effect of the Unr containing c-fos mRNA protein complex to destabilize the c-fos mRNA. The difference may be an effect of other interacting proteins that differ between the two complexes.

Biochemical studies have shown that, in vitro, Unr binds preferentially to purine-rich RNA sequences, present in unstructured regions of RNA (25). Two related consensus sequences, preferentially bound by Unr, were determined. These consensus sequences...
are characterized by a conserved core motif AAGUA or AACG downstream of a purine stretch, and appear unstructured (25). The Unr binding sites of the Apaf-1 and c-fos transcripts are also located in purine-rich regions of their mRNA (22, 23). Of interest is the finding that Unr preferentially binds to single-stranded RNA with a very low affinity for double-stranded RNA. We have previously shown that the PTH mRNA 3′-UTR including the cis-acting element is part of an open single-stranded unstructured region (8). This single-stranded open conformation would favor binding by Unr. Interestingly, the PTH mRNA binding element includes the AAGUA conserved Unr binding core motif that has been reported for the Apaf-1 and c-fos transcripts (25). In addition to rat, this element is present in the PTH mRNA 3′-UTRs of all of the species that have the PTH mRNA cis element (11). Mutations that replaced the first A and the last UA nt of the AAGUA cis-acting element shows that Unr acts through its interaction with the open single-stranded structure of the PTH mRNA 3′-UTR and also prevented the destabilizing effect of the PTH mRNA binding region on GFP reporter gene mRNA in transfection experiments in HEK293 cells (8). Moreover, the truncated rat PTH 3′-UTR RNA probe (11). Mutations that replaced the first A and the last UA nt of the AAGUA cis-acting element shows that Unr acts through its interaction with the open single-stranded structure of the PTH mRNA 3′-UTR and also prevented the destabilizing effect of the PTH mRNA binding region on GFP reporter gene mRNA in transfection experiments in HEK293 cells (8). Moreover, the truncated rat PTH cDNA or a truncated 40-nt PTH element inserted in the GH gene, did not contain the Unr core binding motif and did not respond to Unr (Fig. 4). Unr acts through its interaction with the PTH mRNA cis element. These results together with the open single-stranded structure of the PTH mRNA 3′-UTR and in particular the protein binding region, further support the function of Unr as a PTH mRNA trans-acting factor that is important for the regulation of PTH mRNA stability. However, the role of Unr in the PT can only be substantiated when there is a PTH cell line.

PTH mRNA stability is regulated by calcium and phosphate through differences in binding of a protein-PTH mRNA 3′-UTR complex. Unr and AUFI are part of this PTH mRNA 3′-UTR binding complex that stabilizes PTH mRNA. The binding and stabilization of PTH mRNA by Unr and AUFI and possibly other proteins that are part of the binding complex suggest that they are targets for regulation by calcium and phosphate that will then lead to differences in PTH mRNA stability. How these proteins act together in the PTH mRNA 3′-UTR protein binding complex in the PT, remains to be determined.

MATERIALS AND METHODS

Isolation and Identification of Unr

Affinity purification of PTH mRNA 3′-UTR binding proteins was performed as previously described from rat brain S100 extracts (3) on a heparin-Sepharose and then on a CNBr-activated Sepharose column bound to 200 μg PTH mRNA 3′UTR that had been synthesized in vitro. Fractions were eluted with increasing NaCl concentrations (0.1–1 M), and aliquots were assayed by UV cross-linking gels. Fractions that showed maximal binding were pooled and concentrated using a Centricon 30 filter (Amicon, Beverly, MA). A small sample was run on a 2-D SDS-PAGE, the proteins transferred to a nitrocellulose membrane, and the mRNA-binding proteins identified by Northwestern analysis with PTH 3′-UTR as a 32P-labeled riboprobe or a probe lacking the 3′-UTR. The pooled fractions were run in parallel on a preparative polyacrylamide gel and stained with silver staining. An approximately 100-kDa band was excised from the gel and degraded with the endoprotease LysC, and the peptide products were analyzed by HPLC and microsequenced by Edman degradation.

Northwestern Analysis

Northwestern analysis was performed as described previously (3). Protein extracts were electrophoresed on 2-D sodium dodecyl sulfate-polyacrylamide gels (26) and electrotransferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) that were first stained by Ponceau red. The membranes were presoaked in TBST [10 mM Tris (pH 8), 150 mM NaCl, 0.05% Tween] and then incubated in a buffer containing 10 mM HEPES (pH 7.6), 40 mM KCl, 5% glycerol, 1 mM dithiothreitol (DTT), 0.3 mM phenylmethylsulfonyl fluoride, 0.2% Nonidet P-40, 0.5 mM NaCl, 3 mM MgCl2, 0.1 mM EDTA, and 5 mg/ml BSA for 15 min at room temperature. The membranes were washed twice in TNE buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT], and then incubation was performed in binding buffer [10 mM HEPES (pH 7.6), 150 mM KCl, 5 mM MgCl2, 0.2 mM DTT, 8% glycerol] supplemented with 100 μg/ml ribonuclease-free tRNA (Boehringer Mannheim, Mannheim, Germany) and the 3′-UTR RNA probe (1 × 106 cpm/ml) for 20 min at 37°C and then for 2 h at room temperature. The membranes were washed twice at room temperature for 5 min with TNE buffer, and RNA binding to the protein was visualized by autoradiography.

2-D Gel Electrophoresis

2-D gels were performed as previously described (12) using Ampholine (pH 3.5–10) (Sigma-Aldrich, St. Louis, MO) and 50–100 μg of the rat brain protein sample. For the second dimension, each strip was glued to a 10% SDS-PAGE gel. The proteins were transferred to a 0.45-μm nitrocellulose membrane (Schleicher and Schuell) for 2 h at 4°C in 180 V and 400 mA in transfer buffer. The membranes were stain with Ponceau S and then probed with in vitro-transcribed RNA probes for Northwestern analysis.

UV Cross-Linking Assay

UV cross-linking assay was performed as previously described (1, 3). To measure binding activity of eluates (8) from the RNA column, salt concentrations were normalized (as measured by conductivity) (3). Equivalent aliquots of each fraction were tested. The proteins were incubated with 32P-labeled RNA for the 3′-UTR of the PTH cDNA. After UV cross-linking, the samples were digested by ribonuclease A, fractionated by SDS-PAGE, and autoradiographed.

REMSA

The PTH 3′-UTR RNA probe (5000 cpm) was incubated with microdissected PT S100 cytosolic extracts in a final volume of 20 μl containing 10 mM Tris (pH 7.5), 0.1 mM K-acetate, 5 mM Mg-acetate, 2 mM DTT, 8 U RNasin, 2 μg tRNA, 50 μg heparin, and 1 μg BSA for 10 min at 4°C. Recombinant Unr was prepared as previously described (25). For binding of
rUnr, a different binding buffer was used consisting of 20 mM Tris (pH 7.4), 150 mM NaCl, 25 μg/ml BSA, 10 ng/μl tRNA, 1 mM β-mercaptoethanol, 1 mM PMSF, and 10% glycerol. For supershift experiments, anti-unr rabbit polyclonal antibody (15) or preimmune serum was added after protein RNA incubation, for an additional 30 min at 4°C. In some experiments, the probe was heated to 80°C and then cooled to room temperature to prevent alternative secondary structures before addition of proteins. For competition experiments, unlabeled transcripts were added to the binding reaction at the indicated molar amounts. The samples were run on a native polyacrylamide gel (4% polyacrylamide:bisacrylamide (70:1)) in a cold room. RNA-protein binding was visualized by autoradiography of the dried gels.

RNA Transcripts

Labeled and unlabeled RNA was transcribed from a linearized plasmid construct containing the 3'-UTR of the PTH cdNA subcloned into PCRII plasmid (Invitrogen, San Diego, CA) using an RNA production kit (Promega, Madison, WI) and SP6 RNA polymerase (3). The transcript for the PTH mRNA without the terminal 60 nt of the PTH mRNA 3'-UTR was transcribed as described previously (1). The RNA was used for the affinity column, UV cross-linking, REMSA, and Northern analysis. The unlabeled transcripts were quantified by spectrophotometry at 260/280 and visualized on agarose gels.

Plasmids

pS16-GH expression plasmids that contained the S16 ribosomal protein promoter linked to GH structural gene containing the cis-acting 63-bp fragment or a 40-bp truncated fragment of the PTH mRNA 3'-UTR inserted between the coding region and the 3'-UTR of GH mRNA, were used as previously described (8). The expression plasmid for the rat PTH full-length cdNA and the rat PTH cdNA lacking the terminal 60 bp were prepared by subcloning a PCR fragment amplified with the following primers: forward primer, CTGTATAATGAAACT-CAGGCTTGAAGAA, and reverse primer, TTCATGATCATTA- AAAGAAGAATATATT.

Transient Transfection Experiments

The plasmids for GH and PTH and Unr-FLAG or control (0.5 μg of each DNA per 24-well plate) were transiently cotransfected into HEK293 by calcium phosphate precipitation. Twenty-four hours after transfection, total RNA was extracted by TRI reagent (Molecular Research Center, Cincinnati, OH) and analyzed for GH mRNA by Northern blot. In parallel, cells were collected and proteins were extracted and analyzed for UNR-FLAG by Western blot using an anti-FLAG antibody (Sigma-Aldrich). Expression of cotransfected GFP plasmid was used as a measure of transfection efficiency as assessed by fluorescent microscopy of the cells before extraction. Cotransfection of the expression plasmid for the human PTH gene and the Unr-siRNAs was performed in 24-well plates. The siRNAs (30–120 nM) were cotransfected with the human PTH expression plasmid (0.5 μg) using Lipofectamine 200

Reagent (Invitrogen) according to the manufacturer’s instructions. After 72 h, medium was collected for PTH immunoassay, and cellular proteins were extracted for Western blots or RNA was extracted for Northern blot analysis of human PTH mRNA levels.

Western Blot Analysis

Proteins were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes that were stained with Ponceau red (Sigma-Aldrich). Anti-Unr rabbit polyclonal antibodies (15) were used at a 1:300 dilution. The anti-FLAG M-5 monoclonal antibody was purchased from Sigma-Aldrich.

Statistical Analysis

Results were analyzed by one-way ANOVA with the post hoc Bonferroni multiple comparison test to determine the significance of differences between means. Values of P < 0.05 were considered statistically significant. Results are expressed as mean ± SEM.

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