Dynein light chain binding to a 3′-untranslated sequence mediates parathyroid hormone mRNA association with microtubules

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The 3′-untranslated region (UTR) of mRNAs binds proteins that determine mRNA stability and localization. The 3′-UTR of parathyroid hormone (PTH) mRNA specifically binds cytoplasmic proteins. We screened an expression library for proteins that bind the PTH mRNA 3′-UTR, and the sequence of 1 clone was identical to that of the dynein light chain LC8, a component of the dynein complexes that translocate cytoplasmic components along microtubules. Recombinant LC8 binds PTH mRNA 3′-UTR, as shown by RNA electrophoretic mobility shift assay. We showed that PTH mRNA colocalizes with microtubules in the parathyroid gland, as well as with a purified microtubule preparation from calf brain, and that this association was mediated by LC8. To our knowledge, this is the first report of a dynein complex protein binding an mRNA. The dynein complex may be the motor that is responsible for transporting mRNAs to specific locations in the cytoplasm and for the consequent is asymmetric distribution of translated proteins in the cell.

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Introduction

Serum calcium is maintained with a narrow physiological range due mainly to the action of parathyroid hormone (PTH), which acts together with the biologically active metabolite of vitamin D, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) (1). A 7-transmembranous calcium-sensing receptor (CaSR) on the parathyroid cell recognizes small decreases in serum-ionized calcium to increase PTH secretion (2). A low serum calcium increases not only PTH secretion, but also PTH mRNA levels (3), and if prolonged, parathyroid cell proliferation (4). PTH and $1,25(OH)_2D_3$ share a further level of regulation in that $1,25(OH)_2D_3$ then acts on the parathyroid to decrease PTH gene transcription, thus completing an endocrinological feedback loop (5). Phosphate also regulates the parathyroid, with a low serum phosphate decreasing serum PTH, PTH mRNA levels, and parathyroid cell proliferation (6–9). The effects of calcium and phosphate on PTH gene expression in vivo are both posttranscriptional (6, 10).

The PTH cDNA consists of 3 exons coding for the 5′-untranslated region (5′-UTR; exon I): the prepro region of PTH (exon II) and the structural hormone, together with the 3′-UTR (exon III) (11, 12). The rat 3′-UTR is 239 nucleotides (nt) long out of the 712 nt of the full-length PTH RNA (12). The 3′-UTR is 42% conserved between human and rat, whereas the coding region is 78% conserved (12). We have shown that cytosolic proteins from parathyroid bind to the 3′-UTR of the rat PTH mRNA and regulate mRNA stability (10). Parathyroid proteins from hypocalcemic rats show increased binding to the PTH mRNA 3′-UTR on ultraviolet (UV) cross-linking and RNA electrophoretic mobility shift assays (REMSA), and this protein-RNA binding is decreased with hypophosphatemic parathyroid proteins. These changes in protein-RNA binding correlate with PTH mRNA levels. There is no parathyroid cell line; therefore, an in vitro PTH RNA stability assay was used and showed stabilization of the transcript by hypocalcemic proteins and marked instability with hypophosphatemic proteins (10). These studies indicate that there are instability regions in the PTH mRNA 3′-UTR that are protected by RNA binding proteins. They provide some insight into the posttranscriptional regulation of the PTH mRNA by calcium and phosphate.

Binding of cytoplasmic proteins to the 3′-UTR of mRNAs is known to mediate not only the stability but also intracellular localization of many mRNAs (13–15). We have performed studies to characterize protein-RNA interactions involved in the regulation of the PTH transcript. To identify proteins that bind to the PTH mRNA 3′-UTR we have developed a novel Northwest-

ern method of expression cloning, using an RNA probe to screen for RNA-binding proteins. One clone was found to code for dynein light chain (*Mr 8000*) (LC8), which is part of the cytoplasmic dynein complex (16). LC8 mediated the binding of PTH mRNA to microtubules and may have a role in the intracellular localization of PTH mRNA. Other mRNAs are known to be localized, and this is dependent on regions in their 3′-UTRs interacting with cytoskeletal elements (14, 15). Localization may promote a more efficient use of the RNA template and spatially restricted distribution of the translated protein.

Methods

Plasmid constructs for RNA transcription. RNA probes were transcribed from the rat PTH cDNA in Bluescript II KS (Stratagene, La Jolla, California, USA) in plasmids that had been linearized using different restriction enzymes (10) (see Figure 2a). The template for the full-length RNA was prepared by digestion of the plasmid with *Sma*I, the probe without the 3′-UTR by digestion with *Xba*I, and a probe without the 60 terminal nt by digestion with *Dra*I. The 3′-UTR template was prepared by PCR of the PTH cDNA fragment using 2 oligonucleotides complimentary to the 5′ and 3′ ends of the 3′-UTR and subsequent subcloning into pCRII (Invitrogen Corp., San Diego, California, USA). The 5′ primer, which included the T3 polymerase primer sequence (underlined), was 5′ ATTAACCCTCACTAAAGGGATGCTGACGTATTC 3′. The 3′ primer was 5′ GATCATTAAACTTTA 3′.

The CaSR cDNA is 5.1 kb (2). Transcription of such a large cDNA did not include the whole cDNA and specifically did not include the 3′-UTR. For the CaSR 3′-UTR, we therefore subcloned the 3′-UTR into Bluescript II KS (Stratagene, La Jolla, California, USA) using a fragment obtained by restriction of the BoPCaRI cDNA in pSPORT (GIBCO BRL, Gaithersburg, Maryland, USA) with *Not*I and *Sma*I. The transcription was performed with T3 RNA polymerase.

Radiolabeled RNA probes for REMSA and Northwestern hybridization. Radiolabeled RNA probes were prepared from linearized templates in a transcription reaction as described previously (10). The specific activity of the RNA probe was $0.5–1.0 \times 10^6$ cpm/ng. For competition experiments unlabeled RNA was transcribed similarly in the presence of 1 mM each of the 4 nucleotides. The RNA was quantified by viewing on a 2% agarose gel.

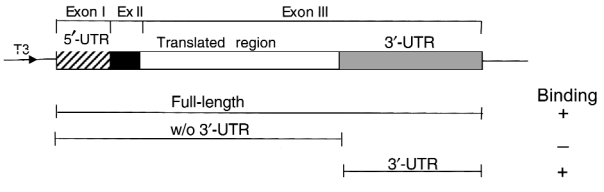


Figure 1
Diagram of PTH cDNA and the PTH mRNA probes used for library screening and protein-RNA binding assays. RNA probes are indicated representing different parts of the PTH mRNA. The binding of LC8 to the RNA probes, as detected by REMSA, is indicated as no detectable binding (–) and positive binding (+).

Northwestern screening of a HeLa expression library (λTriplEx). The library was plated and plaques were transferred to nitrocellulose filters (Schleicher & Schuell GmbH, Dassel, Germany) according to the manufacturer's instructions (CLONTECH Laboratories Inc., Palo Alto, California, USA). To identify positive clones expressing proteins binding to the PTH mRNA, the membranes were hybridized to an RNA probe representing the full-length PTH, or a probe lacking the 3′-UTR, or a probe representing only the 3′-UTR (10). This Northwestern hybridization was performed by incubating the presoaked membranes (2× SSC) in prehybridization buffer (10 mM HEPES, pH 7.6, 40 mM KCl, 5% glycerol, 1 mM DDT, 0.3 mM PMSF, 0.2% NP40, 0.5 M NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 5 mg/mL BSA) for 15 minutes 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 the hybridization buffer (10 mM HEPES, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM DTT, 8% glycerol), supplemented with 5 mg/mL RNase free tRNA from *Escherichia coli* (Boehringer Mannheim GmbH, Mannheim, Germany), and the RNA probe (2×105 cpm) was added for 20 minutes at 37°C, then for 2 hours at room temperature. The membranes were washed twice at room temperature for 5 minutes with TNE buffer and the RNA-binding clones were viewed using autoradiography. Phages from positive plaques were replated for additional screening. At the fourth screening all the plaques were positive, indicating a homogenous clone. Phages from a positive plaque were isolated and converted to plasmids. Plasmid DNA was sequenced using primers provided with the library. The DNA and protein sequences were used to search the National Institutes of Health (NIH; Bethesda, Maryland, USA) and GCG Genebank (Madison, Wisconsin, USA) databases.

Northern blot analysis. Total RNA was extracted from rat parathyroid tissue and cell lines by Tri-reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA) and analyzed for mRNA levels as before (6), with random-primed cDNAs for PTH and the cloned fragment from the expression library. For the microtubule-association experiments, Northern blot analysis was performed with cDNAs for PTH, the CaSR, and ribosomal RNA.

Western blot analysis for LC8 and dynein intermediate chain. Protein extracts were electrophoresed on SDS-10% polyacrylamide gels (17) and electrotransferred onto nitrocellulose membranes (0.2 μm, Schleicher & Schuell, Keene, New Hampshire, USA). The resulting blots were blocked at room temperature (1 hour) with PBS containing 5% powdered skimmed milk and 0.3% Tween-20 (blocking buffer). The blots were allowed to react overnight at 4°C with blot-purified rabbit anti–dynein light chain (anti-Dlc) LC8 antibody (1:500), which recognized the denatured form of LC8 (R4058) (18). For the dynein intermediate chain 74 (IC

74), antibody 74-1 was used (18). Blots were washed in PBS containing 0.3% Tween-20 and incubated (1 hour) with peroxidase-conjugated Affinipure goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) 1:7,500 in PBS containing 1% BSA. After washing, the blots were soaked in Renaissance Western blot chemiluminescence detection system (1 minute) (DuPont-NEN, Boston, Massachusetts, USA) and briefly dried. Immunoreactive bands were viewed using light emission on Kodak film.

REMSA. RNA probe (1.5×10^4 cpm) was incubated with 0.4 μg of recombinant LC8 (16) in a final volume of 20 μL containing 10 mM HEPES, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, and 1 mM DTT (binding buffer). After 20 minutes at room temperature, RNase T1 (Sigma Chemical Co., St. Louis, Missouri, USA) was added for 10 minutes at room temperature to a final concentration of 150 U/mL to digest unprotected RNA. The samples were then loaded on a polyacrylamide electrophoresis gel (40% acrylamide, 70:1) and run in a cold room. RNA-protein binding was viewed using autoradiography. In some experiments proteinase K (1 mg/mL) was added for 10 minutes at 37°C. For competition experiments unlabeled RNA was added.

RNA cosedimentation with polymerized tubulin. The microtubule protein preparation, consisting of tubulin together with microtubule-associated proteins, was extracted from calf brains according to our published methodology (19). One hundred micrograms of tubulin preparation (20 μM) in a volume of 50 μL were polymerized in vitro by the addition of 20 μM paclitaxel (Sigma), 1 mM GTP, and 30% glycerol, and incubated at 37°C for 10 minutes. Total RNA was extracted from rat thyroparathyroid tissue by Tri-reagent (Molecular Research Center Inc.) (6), 2 μg of total RNA was added to the assembled microtubules (paclitaxel, GTP) and incubated at room temperature for 20 minutes to allow binding. The samples were then placed over a 200-μL 30% sucrose cushion and pelleted by centrifugation at 40,000 *g* for 1 hour at 20°C. In some experiments, recombinant LC8 (0.2, 0.4, or 2 μg) was added to the microtubule preparation with the RNA. RNA was extracted from the pellets and supernatants by Tri-reagent and analyzed by Northern blot analysis for PTH mRNA (6). In other experiments, RNA for PTH or the 3′-UTR of the CaSR was transcribed in vitro, and 10 ng of RNA was incubated with the polymerized microtubule preparation and sedimented, extracted, and analyzed by Northern blot analysis as above. Assembly of microtubules was determined periodically at 350 nm. In addition, aliquots of the pellet and supernatant were analyzed by negative-staining electron microscopy to confirm the assembled polymerized (paclitaxel, GTP) microtubules.

The association of endogenous PTH mRNA to microtubules from the rat parathyroid was also studied. Thyroparathyroid tissue from 6 rats was homogenized in MES buffer (1 mM) in the presence of 200 U of RNase inhibitor (Promega Corp., Madison, Wisconsin, USA) and 0.5 mM PMSF and then centrifuged at 40,000 *g* for 40 minutes at 4°C. The acceleration was chosen to allow precipitation of organelles and cytoskeletal structures without precipitation of soluble elements such as tubulin in either its monomeric or ring structure. The supernatant was supplemented with 30% glycerol and divided into 3 samples. Paclitaxel (20 μM) and GTP (1 mM) were added to 2 samples. ATP (5 mM) and NaCl (1 M) were then added to one of these samples. No paclitaxel, GTP, ATP, or NaCl were added to the third sample. In another experiment ATP and NaCl were added separately, with paclitaxel and GTP. The samples were incubated for 20 minutes at 37°C for polymerization of the microtubules and then centrifuged through a 30% sucrose cushion as before. RNA was extracted from the pellet and the supernatant and was analyzed by a Northern blot analysis gel for PTH and 18S RNA.

Immunoprecipitation of intermediate chain from parathyroid extracts and RNA analysis. Thyroparathyroid tissue from 6 rats was homogenized in lysis buffer (50 mM Tris, pH 8.1, 5 mM EDTA, 150 mM NaCl, 1% Triton-X 100, RNase inhibitor, 0.5 mM DTT, 0.1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A). The homogenate was centrifuged at 4,000 *g* for 15 minutes at 4°C, and the recovered supernatant was respun. Protein A-Sepharose beads (Sigma) were washed and incubated while rocking with IC antibody or preimmune serum for 1 hour at 4°C. The beads were then washed 3 times with PBS and added to the precleared parathyroid extract with 150 μg/mL of yeast tRNA (Roche Molecu-

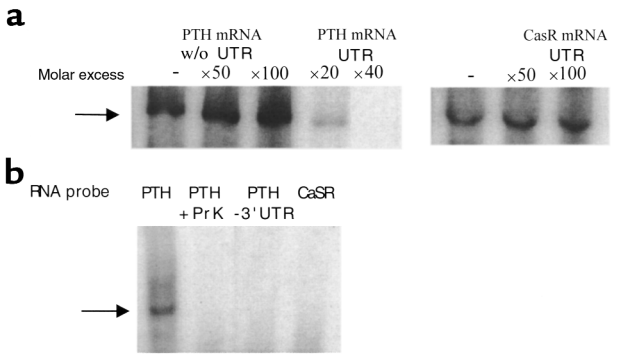


Figure 2
LC8 binds PTH mRNA and not the CaSR 3′-UTR mRNA. (**a**) RNA REMSA for the full-length PTH RNA. Labeled transcripts for the full-length PTH RNA were incubated with recombinant LC8 and then treated with RNase T1 and run on a native PAGE. There was a single protein-RNA band. Increasing concentrations of unlabeled PTH mRNA without the 3′-UTR did not compete for binding. The PTH mRNA 3′-UTR in excess competed for protein RNA binding even at a low concentration of the unlabeled RNA, indicating specificity. Excess CaSR 3′-UTR mRNA did not affect binding of the full-length PTH mRNA to LC8. (**b**) REMSA for the 3′-UTR of the PTH RNA and the CaSR. Recombinant LC8 was incubated with the different PTH mRNA probes and the CaSR 3′-UTR mRNA probe and samples, treated with RNase T1, and run on a nondenaturing acrylamide gel. The REMSA showed that LC8 binds the PTH mRNA 3′-UTR, and this binding was not present after treatment with proteinase K. A probe lacking the PTH mRNA 3′-UTR and a probe for the CaSR 3′-UTR mRNA showed no binding. The arrow indicates the protein RNA complex.

lar Biochemicals, Basel, Switzerland) and rocked at 4°C for 3 hours. The parathyroid extract had been precleared by incubation with prewashed protein A beads in the presence of 60 μg/mL of yeast tRNA (Roche Molecular Biochemicals) at 4°C for 30 minutes. RNA was extracted with Tri-reagent (Molecular Research Center, Inc.) from the immunoprecipitated bead pellet after elution with wash buffer (125 mM Tris, pH 8.1, 1 M NaCl, 5 mM EDTA, 0.02% NaN_3) and addition of 10 μg of yeast tRNA (Roche Molecular Biochemicals). The RNA was run on a gel, blotted, viewed using ethidium bromide staining, and hybridized for PTH and H4 histone as described previously (6).

Results

We have shown that cytoplasmic proteins from parathyroid bind to the 3′-UTR of the rat PTH mRNA (10). These proteins are present in other rat tissues, such as brain, liver, and muscle, as well as other species, such as human and bovine (10). We therefore screened a HeLa cell cDNA phage-expression library for proteins that bind the full-length rat PTH mRNA, but not a probe for the PTH mRNA lacking the 3′-UTR (Figure 1). To do this, the library was plated and transferred to membranes, which were hybridized to the labeled PTH RNA transcripts. This expression cloning revealed 15 clones that bind the full-length PTH mRNA; only 3 of these clones were specific to the 3′-UTR and did not bind a probe lacking the 3′-UTR. These results indicate that the 3′-UTR was the binding site for the expressed proteins. One clone was further characterized because upon sequencing, it was found to be identical to the COOH-terminal third of Dlc (M_r*8000*) LC8. The insert of this clone was used as a probe for Northern blot analysis. There was a single transcript of 700 nt, which is the predicted size of mammalian Dlc (M_r*8000*) cDNA that was expressed in rat parathyroid glands, as well as in other tissues and cell lines, such as HeLa cells (T47D, breast

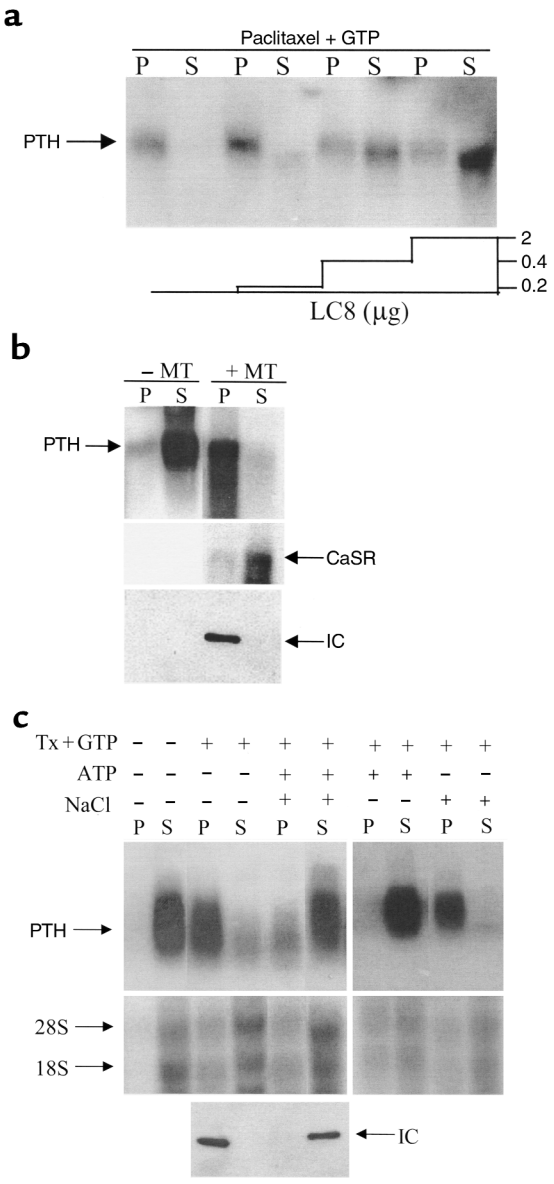


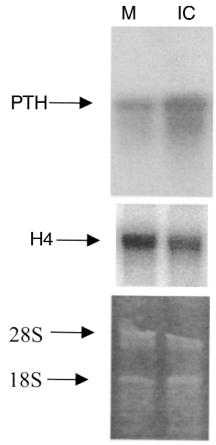
Figure 3
PTH mRNA colocalization with microtubules is dependent upon LC8. (**a**) Purified calf brain microtubule preparations, which contained MAPs, were assembled in the presence of paclitaxel and GTP and incubated with total RNA from rat thyroparathyroid tissue. Polymerized microtubules were sedimented, and the RNA, which was extracted from both the supernatants (S) and the pellets (P), was assayed for PTH mRNA by Northern blot analysis. PTH mRNA was present in the polymerized microtubule pellet and not in the supernatant. When increasing concentrations of LC8 were added to the RNA-microtubule preparation, there was a shift of PTH mRNA from the pellet to the supernatant, indicating that LC8 mediates the association of PTH mRNA to microtubules. (**b**) In vitro transcribed RNA for PTH or the CaSR 3′-UTR were incubated either without (–) or with (+) polymerized calf brain microtubule preparations that contained MAPs, and the samples were centrifuged to separate the microtubule pellet (P) from the supernatant (S). The RNA from both fractions was analyzed by Northern blot analysis (top 2 panels). When no microtubules were added, PTH mRNA was in the supernatant. With polymerized microtubules, PTH mRNA was present in the microtubule pellet, and the CaSR 3′-UTR RNA was present in the supernatant. Western blot analysis of samples of each fraction with antibody 74-1 to dynein IC 74 is shown in the bottom panel. IC was present in the microtubule pellet. (**c**) Cytosolic protein extracts derived from rat thyroparathyroid tissue were incubated without or with paclitaxel and GTP to polymerize endogenous microtubules, with or without ATP (5 mM) and NaCl (1 M), and the samples were centrifuged to separate the microtubule pellet (P) from the supernatant (S). The samples were then examined for PTH and ribosomal RNA using Northern blot analysis. In the absence of paclitaxel and GTP, both PTH and ribosomal RNA (28S and 18S) were present in the supernatant. When the microtubules were polymerized by the addition of paclitaxel and GTP, the PTH RNA was present in the microtubule pellet and the ribosomal RNA was still present in the supernatant. Addition of ATP and NaCl, or ATP alone, which specifically elute the dynein from the microtubules, displaced PTH mRNA to the supernatant. Addition of NaCl alone had no effect. This indicates that PTH mRNA associates with dynein in an ATP-dependent manner. Western blot analysis as in **b** showed that IC was present in the microtubule pellet and was eluted by the addition of ATP and NaCl.

cancer cell line) and HEK (human embryonic kidney) 293 cells (not shown). Western blot analysis showed the expression of LC8 protein in the parathyroid as well as in other tissues such as brain (not shown).

To demonstrate the binding of LC8 to the PTH RNA, recombinant LC8 was run on a denaturing gel, then transferred to a membrane and hybridized with an RNA probe for the PTH mRNA. Surprisingly, this Northwestern screening gel showed no binding (not shown). However, the Northwestern gel uses a denaturing gel; in the Northwestern screening that we used for identifying LC8 as a PTH RNA-binding protein, the proteins retain their native structure because they are lifted from the colonies directly with no denaturation. This suggested that the native structure of the protein was important to the binding of PTH mRNA. We therefore characterized the binding of LC8 to PTH mRNA under nondenaturing conditions, which would be similar to the conditions in which LC8 was cloned using the expression library. To do this, we performed REMSA. We incubated recombinant LC8 with the different PTH mRNA probes (Figure 1), digested the mixture with RNase T1 to digest unbound RNA, and then ran the samples on a native gel. The REMSA showed that the full-length PTH transcript (Figure 2a) and the 3′-UTR RNA (Figure 2b) both bound LC8 with a single band of the same size. The specificity of the binding was demonstrated in competition experiments (Figure 2a). Binding of the full-length PTH mRNA probe was not competed for by excess PTH mRNA, which excluded the 3′-UTR (Figure 2a). Binding was markedly decreased by excess cold 3′-UTR (Figure 2a), as well as by excess full-length PTH mRNA (not shown). This indicates that the 3′-UTR of the RNA contains the binding site to LC8. The 3′-UTR RNA of another gene, CaSR (2), did not compete for binding of the full-length PTH mRNA probe to LC8 (Figure 2a). When no protein was added, there was no band detected, indicating that the RNA probe was totally digested by RNase T1 in the absence of protein (not shown). Figure 2b shows the binding to the 3′-UTR probe for the PTH mRNA. Proteinase K eliminated the binding (Figure 2b) and also the binding of the full-length probe (not shown) indicating that a protein, LC8, protected the mRNA probe. The restricted PTH mRNA probe without the 3′-UTR showed no binding (Figure 2b). An RNA probe for the CaSR 3′-UTR also did not bind LC8 (Figure 2b). A transcript for the coding region of the CaSR also showed no binding (not shown). These gels show that LC8 specifically binds to the PTH mRNA 3′-UTR. To delineate the protein-binding region in the 3′-UTR, a PTH RNA transcript that excluded the terminal 60 nt of the 239 nt of the 3′-UTR was analyzed for binding to LC8 by REMSA and showed no binding (not shown). We have shown previously that this terminal 60-nt region is important for the interaction of parathyroid proteins with the PTH mRNA and for PTH mRNA stability (10).

LC8 is part of the dynein complex, which is a molecular motor that translocates along microtubules (20, 21). Because LC8 binds PTH mRNA, we asked whether LC8 mediates an interaction between the PTH mRNA and microtubules. We therefore studied the binding of PTH mRNA to microtubules and its competition by LC8. A calf brain microtubule preparation that contained microtubule-associated proteins (MAPs) was incubated with paclitaxel and GTP to facilitate polymerization of the tubulin subunits to microtubules, or without paclitaxel and GTP. The polymerized and unpolymerized microtubules were incubated with total RNA from rat thyroparathyroid tissue to allow binding. The samples were then loaded onto a sucrose cushion and centrifuged to separate the pellet containing the polymerized microtubules and associated proteins from the supernatant. RNA was extracted from the pellet and the supernatant, and a Northern blot analysis was conducted to identify PTH mRNA. Without paclitaxel and GTP, the PTH in microtubules from parathyroids, was in the supernatant of the brain microtubule preparation (not shown). In the presence of paclitaxel and GTP, the PTH mRNA was present in the microtubule pellet and not in the supernatant (Figure 3a). When increasing concentrations of exogenous LC8 were added to the RNA-polymerized microtubule preparation there was a gradual shift of PTH mRNA from the pellet to the supernatant (Figure 3a). Western blot analysis of samples from the pellet and supernatant with antibody to LC8 (R4058; ref. 18) showed that the endogenous LC8 was present in the polymerized microtubule pellet and not in the supernatant (not shown). Addition of LC8 did not change the amount of LC8 in the pellet of all the samples and also had no effect on microtubule polymerization, as viewed using electron microscopy (not shown). These results indicate that the translocation of the PTH mRNA from the microtubule pellet to the supernatant was not due to an effect of LC8 on microtubule disruption but rather as a result of competitive binding. The LC8 was added to the microtubule preparation at a ratio of 1:500 (by weight). The ratio of endogenous LC8 to microtubules is not known. Added LC8 in increasing amounts had a

Figure 4
PTH mRNA was immunoprecipitated with IC antibodies. Parathyroid extracts were incubated with preimmune mock (M) or IC antibodies and RNA was eluted from the pellets and analyzed for PTH mRNA and H4 histone. The exposure time was 1 day for PTH mRNA and 4 days for H4 histone mRNA. Ethidium bromide staining of the membranes is shown in the bottom panel. There was more PTH mRNA in the IC immunoprecipitate than in mock antibody.



dose-dependent effect to translocate the PTH mRNA from the microtubule pellet to the supernatant. This suggests that the amount of LC8 protein added was of functional relevance to the amount of endogenous LC8 present in the microtubule preparation. When another protein, BSA, was added to the microtubule assay at similar concentrations to LC8, there was no shift of the PTH mRNA to the supernatant (not shown). The colocalization of PTH mRNA with the polymerized microtubule preparation pellet and its shift to the supernatant after the addition of increasing amounts of LC8, shows that PTH mRNA binds microtubules and that LC8 competes for this binding. These results suggest that LC8 is important for the interaction of microtubules with PTH mRNA.

In other experiments, RNA was transcribed in vitro, using templates for full-length PTH cDNA and the cDNA for the CaSR and added to the microtubule preparation as before (Figure 3b). In the absence of microtubules and after centrifugation, the PTH transcript was found in the supernatant as expected (Figure 3b). After incubation of the PTH transcript with polymerized microtubules (paclitaxel and GTP), the PTH RNA, but not the CaSR RNA, was present in the microtubule pellet and not in the supernatant (Figure 3b). Western blot analysis of the samples was performed using an antibody (IC 74-1) for IC 74 of the cytoplasmic dynein (18). Quantitative stoichiometry has demonstrated that there is 1 copy of the LC8 polypeptide per IC 74 (18). The polymerized microtubule complex (P in Figure 3b) contained the dynein IC together with the full-length PTH RNA. When incubated with unpolymerized tubulin (no paclitaxel and GTP), the PTH transcript and the IC were present in the supernatant (not shown). The IC is an integral part of the dynein complex, of which LC8 is a part, and IC therefore reflects the localization of the dynein complex. These results show that the colocalization of the PTH mRNA with the polymerized microtubule pellet was the same when total RNA from parathyroid (Figure 3a) or in vitro transcribed full-length PTH RNA (Figure 3b) was incubated with polymerized microtubules. When PTH transcripts that excluded the 3′-UTR or the terminal 60 nt were added to polymerized microtubules at the same ratio as controls, the transcripts were present in the supernatant and not in the microtubule pellet (not shown). Another transcript, the CaSR RNA, was used because it is also highly expressed in the parathyroid (2). This transcript is 5,000 nt long; therefore, we transcribed separately transcripts for the first approximately 3,000 nt, which include most of the coding region and transcripts for the terminal approximately 1,500 nt, which includes the intact 3′-UTR. Transcripts for both the CaSR coding region (not shown) and 3′-UTR that did not bind LC8 (Figure 2b) were present in the supernatant and not in the pellet (Figure 3b). The binding to polymerized microtubules of full-length PTH mRNA and not the CaSR mRNA 3′-UTR suggests that the ability to bind LC8 is important to this interaction.

To show in vivo that the PTH mRNA localized with the microtubule fraction in the thyroparathyroid tissue, paclitaxel-stabilized cytosolic extracts of thyroparathyroid tissue were sedimented to separate the pellet from the supernatant, as before. RNA was extracted from both fractions and analyzed for PTH mRNA using Northern blot analysis. When there was no polymerization of the microtubule fraction (no paclitaxel and GTP added to the cytosolic extract), all the PTH mRNA was present in the supernatant (Figure 3c). After polymerization with paclitaxel and GTP, the PTH mRNA was found predominantly in the microtubule pellet of the parathyroid (Figure 3c). To show that the colocalization of the PTH RNA with the polymerized microtubule pellet was mediated by the dynein complex, we added ATP (5 mM) and NaCl (1 M) to the parathyroid microtubule preparation, which is known to lead to dissociation of dynein from microtubules (18, 22). Addition of ATP and NaCl resulted in a shift of the PTH mRNA and the dynein complex from the pellet to the supernatant (Figure 3c). Western blot analysis of the pellet and supernatant samples with IC antibodies showed that the dynein complex colocalized with the PTH mRNA and was eluted by ATP and NaCl (Figure 3c). A high NaCl concentration itself may disrupt binding to other components of the extract. We therefore added ATP and NaCl separately. ATP alone led to a shift of PTH mRNA from the pellet to the supernatant (Figure 3c). The addition of NaCl alone had no effect upon the association of PTH mRNA with the microtubule preparation. All the PTH mRNA remained in the pellet (Figure 3c). Therefore, the dissociation of PTH mRNA from polymerized microtubules was ATP dependent and not NaCl dependent. This suggests that the effect is not dependent upon binding to actin, intermediate filaments, membranes, or nonspecific aggregates. The membranes were rehybridized for ribosomal RNAs as controls. With and without microtubule polymerization, ATP, and NaCl, ribosomal RNA was present mainly in the supernatant of the cytosolic preparation (Figure 3c), indicating that the colocalization of PTH mRNA with the microtubule pellet was specific. The results of Figure 3, a–c, indicate that PTH mRNA is associated with microtubules both in vivo and in vitro and suggest that LC8 may mediate this association.

To demonstrate that PTH mRNA is bound to dynein in vivo in the parathyroids, we immunoprecipitated the dynein complex with an antibody to IC (74-1 mAb) and studied the presence of PTH mRNA in the immunoprecipitate. The LC8 protein is buried mostly within large macromolecular complexes with which it associates, and therefore antibodies to LC8 cannot gain access to it. For this reason we immunoprecipitated cytoplasmic dynein from the extract by using the IC antibody. As a control, preimmune serum (mock) was used. The antibodies, attached to protein A-

agarose beads, were incubated with parathyroid protein homogenates. The beads were precipitated and the attached RNA eluted and extracted for analysis. Northern blot analysis for PTH mRNA was performed. Eluants from the pellets immunoprecipitated with both IC antibody and preimmune serum bind RNA as seen in Figure 4. However, PTH mRNA was precipitated appreciably more by IC antibody than by mock antibody (Figure 4). Rehybridization of the membrane for H4 histone and ethidium bromide staining of the membrane showed binding of these RNAs to both antibodies, but there was no difference in binding between the specific and mock antibodies (Figure 4). It is not readily explainable why the RNAs were precipitated by both antibodies. However, IC antibody showed increased binding only to the PTH mRNA and not to ribosomal RNAs. This result suggests that PTH mRNA specifically associates with the dynein complex in the parathyroid. This binding may mediate the association of PTH mRNA with the microtubules.

Discussion

The binding of cytoplasmic proteins to the 3′-UTR of mRNAs has been associated with many properties of the mRNA, such as its stability, translation, and intracellular localization. To identify proteins that bind to the PTH mRNA 3′-UTR, expression cloning was performed using a Northwestern strategy. This expression cloning identified 3 clones that bind the full-length PTH mRNA and the 3′-UTR but did not bind a probe lacking the 3′-UTR. One of these clones was identified by sequencing to be LC8. LC8 mRNA and protein were expressed in the parathyroid. The binding of LC8 to the PTH mRNA 3′-UTR was confirmed by the binding of recombinant LC8 to the full-length PTH mRNA and 3′-UTR transcripts by REMSA. LC8 is part of the dynein complex of the cell that binds to microtubules (20). We showed that PTH mRNA from rat parathyroids associated in vitro with polymerized microtubules prepared from calf brains, and this association was competed for by excess LC8. In addition, endogenous PTH mRNA also associated with polymerized microtubules prepared from a cytosolic extract of rat parathyroid glands. The presence of the dynein complex in the microtubule pellet was confirmed by Western blotting with antibodies against components of the dynein complex. To demonstrate the interaction of PTH mRNA and the dynein complex in extracts of the parathyroid glands, we immunoprecipitated the dynein complex by an antibody to IC and measured the presence of PTH mRNA in this immunoprecipitate. Immunoprecipitation by IC antibody contained more PTH mRNA than the mock immunoprecipitation. Suggesting that PTH mRNA interacts with the dynein complex in vivo, rRNA was not immunoprecipitated preferentially by the IC antibody.

The association of PTH mRNA with LC8 and microtubules suggests that the parathyroid cell uses LC8 to bind the PTH mRNA and translocate it along microtubules for its further processing or translation. The size of LC8 (8 kDa) is not compatible with the size of the PTH RNA-binding proteins (50, 60, and 110 kDa) (10) whose binding is regulated by calcium and phosphate and determines RNA stability. It is interesting that the binding of these proteins is also dependent upon the terminal 60 nt of the PTH mRNA 3′-UTR, as it is for LC8 (10). This suggests that this region is functionally important. LC8 may regulate PTH mRNA localization but its contribution to PTH mRNA stability is not known.

There are many other examples of mRNAs localizing to specific regions in a cell. Localization of mRNAs in other systems is related to the interaction of their 3′-UTRs with proteins, which bind cellular elements (14, 15). In *Drosophila* embryogenesis, proper intracellular localization of bicoid and nanos mRNAs is critical for generating the embryonic body plan (23). In *Xenopus* oocytes, Vg1, a TGF-β homologue that induces mesoderm formation, is localized to the vegetal cortex (24). The cellular localization of mRNAs is thought in some cases to involve transport along microtubules (25, 26). In neuronal cells there is localization of tau mRNA, which is dependent on binding to microtubules and not to actin (27), and tau protein itself stabilizes microtubules (28). Vg1 mRNA binds Vg1 RNA-binding protein, which mediates its association with microtubules (29). RNA moving down the microtubule has been successfully shown for myelin basic protein (MBP) mRNA. Ainger et al. injected fluorescently labeled MBP mRNA into cultured oligodendrocytes and showed that mRNA accumulated in particles, which then moved in an anterograde direction into the cell processes, probably along microtubules (30, 31). These granules not only contained MBP mRNA but also components needed for protein translation (32). This spatial organization would increase the efficiency of protein synthesis.

Intracellular localization has been shown for other hormones. Vasopressin and oxytocin are synthesized in the supraoptic and paraventricular magnocellular nuclei in the hypothalamus, and the translated products are then transported in neurosecretory granules down the axons and stored in nerve terminals in the pars nervosa of the pituitary. In addition, it has been shown that vasopressin and oxytocin transcripts are also present in the posterior pituitary and that these transcripts are the products of primary transcripts in the hypothalamus that have been transported along axons (33).

The development of the fetal ductus arteriosus is dependent on smooth muscle migration that is associated with greater efficiency of fibronectin mRNA translation, and this has been shown to be dependent upon the binding of an element in the fibronectin mRNA 3′-UTR to microtubule-associated protein 1 light chain 3 (34). The binding of PTH mRNA to LC8 was shown here to be specific to sequences in its 3′-UTR. LC8 is part of the dynein motor system of the cell that is involved classically in minus end-directed movement

along microtubules (18). The orientation of the parathyroid microtubules is not known. In other systems LC8 has a role in transporting cargoes to which they are attached along microtubules, but it also has other regulatory roles in the cell. For instance, LC8 inhibits neuronal nitric oxide synthase by interacting with the nitric oxide synthase dimer, causing it to dissociate into inactive monomers (35). In addition, LC8 interacts with Bim, a member of the Bcl-2 family, to localize and regulate Bim's proapoptotic activity (36).

We have now identified LC8 by differential Northwestern expression screening with the PTH 3′-UTR. We have screened an expression library from the brain but have shown that the LC8 protein is expressed in the parathyroid and mediates interaction of PTH mRNA with microtubules in vitro and in vivo in parathyroid cytosolic extracts. To our knowledge, this is the first demonstration that LC8 binds a mRNA and mediates the association of a mRNA with microtubules. Further studies will determine whether PTH mRNA interaction with LC8 and microtubules is used by the cell to efficiently process and translate PTH mRNA.

Note added in proof: We have now isolated, by affinity chromatography, another protein, AUF1, that binds to the PTH mRNA3′-UTR. AUF1 has a functional role in determining the stability of the PTH transcript in response to changes in serum calcium and phosphate. (A. Sela-Brown, J. Silver, G. Brewer, and T. Naveh-Many, 2000, *J. Biol. Chem.*, in press).

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