Protein-RNA Interactions Determine the Stability of the Renal NaPi-2 Cotransporter mRNA and Its Translation in Hypophosphatemic Rats*

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Hypophosphatemia leads to an increase in type II Na\(^{+}\)-dependent inorganic phosphate cotransporter (NaPi-2) mRNA and protein levels in the kidney and increases renal phosphate reabsorption. Nuclear transcript run-on experiments showed that the effect of a low phosphate diet was post-transcriptional. In an \textit{in vitro} degradation assay, renal proteins from hypophosphatemic rats stabilized the NaPi-2 transcript 6-fold compared with control rats and this was dependent upon an intact NaPi-2 3'-untranslated region (UTR). To determine an effect of hypophosphatemia upon NaPi-2 protein synthesis, the incorporation of injected \(^{35}\)S)methionine into renal proteins was studied \textit{in vivo}. Hypophosphatemia led to increased \(^{35}\)S)methionine incorporation only into NaPi-2 protein. The effect of hypophosphatemia on translation was studied in an \textit{in vitro} translation assay, where hypophosphatemic renal proteins led to increased translation of NaPi-2 and other transcripts. NaPi-2 RNA interaction with cytosolic proteins was studied by UV cross-linking and Northwestern gels. Hypophosphatemic proteins led to increased binding of renal cytosolic proteins to the 5'-UTR of NaPi-2 mRNA. Therefore, hypophosphatemia increases NaPi-2 gene expression post-transcriptionally, which correlates with a more stable transcript mediated by the 3'-UTR, and an increase in NaPi-2 translation involving protein binding to the 5'-UTR. These findings show that phosphate regulates gene expression by affecting protein-RNA interactions \textit{in vivo}. 

Dietary phosphorus is converted in the body to the phosphates, in which form it exerts its widespread physiological functions, as an essential component of phospholipids, ATP, DNA, phosphorylated proteins, metabolic intermediaries, body buffers, and bone (1). The renal tubule has an intrinsic ability to adjust the reabsorption rate of phosphate according to the need and availability of phosphate to the body. The renal tubule responds to a decrease in filtered phosphate load with an increase in transport activity, thereby maintaining renal phosphate homeostasis. The active reabsorption is mediated by the Na\(^{-}\)-dependent inorganic phosphate cotransporters (NaPi).\(^1\) NaPi type II are expressed at the apical brush border membranes of the proximal tubules and are predominantly responsible for the regulated reabsorption of phosphate in response to changes in dietary phosphate (2–4). In the rat, NaPi type II is termed NaPi-2. Deletion of the NaPi-2 gene in mice leads to severe phosphate wasting (5). The activity of NaPi-2 is increased by a low phosphate diet (6–8) and after parathyroidectomy (9) (10) and decreased by a high phosphate diet, parathyroid hormone (9), glucocorticoids (11, 12), and metabolic acidosis (13, 14). The increase in NaPi-2 is the result of an increase in \(V_{\text{max}}\) suggesting an increase in the number of apical NaPi-2 transporters, by a transporter shuffling mechanism, which is sensitive to disruptors of microtubule integrity (13, 15). A low phosphate diet for as little as 2 h led to an increased transfer of preformed NaPi-2 from the endosome to the apical membrane by a microtubule-dependent mechanism (16). Chronic hypophosphatemia increases renal NaPi-2 activity not only by increasing transfer to the apical membrane (9) but also by increasing the levels of its mRNA as well as the amount of the cotransporter protein (10). The adaptive response to extracellular Pi deprivation also occurs \textit{in vitro} in opossum kidney cells, where it has been shown to be a post-transcriptional effect (17, 18). The decrease in NaPi-2 cotransporter activity caused by PTH is associated with endocytosis of brush border membrane-associated NaPi-2 protein and is not accompanied by a decrease in its mRNA levels (19, 20).

We have now studied \textit{in vivo} in the rat how chronic hypophosphatemia increases the expression of NaPi-2 mRNA and protein in the kidney. The results show that the effect on NaPi-2 mRNA is post-transcriptional. An \textit{in vitro} degradation assay showed that the NaPi-2 transcript was more stable in the presence of renal proteins from hypophosphatemic rats than proteins from controls. RNA stabilization by hypophosphatemic proteins was mediated by the 3'-UTR of the NaPi-2 transcript. Hypophosphatemic renal proteins, but not hepatic proteins, showed increased binding to the NaPi-2 5'-UTR by UV cross-linking and Northwestern gels. \textit{In vivo}, the incorporation of injected \(^{35}\)S)methionine into renal NaPi-2 protein was increased by a low phosphate diet. We demonstrated that kidney cytosolic proteins from hypophosphatemic rats led to increased synthesis of NaPi-2 in an \textit{in vitro} translation assay, suggesting an increase in translation in addition to the increased mRNA levels.

**EXPERIMENTAL PROCEDURES**

**Animals**—Weanling male Sabra rats were fed a normal phosphate (0.3%), normal calcium (0.6%), or a low phosphate (0.02%) normal calcium (0.6%) diet (Teklad) for 2 weeks. This low phosphate diet resulted in a serum phosphate of 4.2 ± 0.4 mg/dl (control = 9.7 ± 1.0 mg/dl) and serum calcium of 12.4 ± 0.7 mg/dl (control = 10.6 ± 0.5 mg/dl). After 2 weeks, the kidneys, liver, and parathyroid glands were removed under pentobarbital anesthesia and blood samples were taken for measurements of serum calcium and phosphate in a Roche autoanalyzer. Tissues for nuclear run-on assays and protein extracts were used immediately as described below.
FIG. 1. The rat NaPi-2 cDNA constructs and the restriction enzymes used for in vitro synthesis of RNA transcripts. A, the full-length NaPi-2 cDNA transcript was used to transcribe the full-length cDNA after linearization of the plasmid with NotI; a 2065-nt transcript excluding the 3′-UTR with NcoI; a 1043-nt transcript, which included the 5′-UTR and only part of the translated region, and excluded the 3′-UTR, with NcoI; and a 53-nt transcript consisting only of the 5′-UTR, with BspHI. B, subcloning of the NaPi-2 3′-UTR into Bluescript KS. The 3′-UTR was transcribed with T3 RNA polymerase after linearization of the plasmid with NotI. The transcript length (nt) is denoted under the restriction sites.

Nuclear Run-on Transcription Assay—Nuclei were prepared from kidney cortex of one rat in each experiment and nuclear run-on transcription assays performed as described previously (21, 22). Elongated 32P-labeled RNA was extracted by TRI reagent (Molecular Research Center, Cincinnati, OH) and resuspended in 300 μl of hybridization buffer (7% SDS, 10% polyethylene glycol 8000, 1.5% saline/sodium phosphate/EDTA). Aliquots of RNA from treated and untreated samples were counted in a scintillation counter, and an equal number of counts from each condition (1–2 × 106 cpm) was hybridized to linearized cDNAs (5 μg) for, parathyroid (PTH)/parathyroid-hormone related protein receptor, NaPi-2, the calcium sensing receptor, actin, and βPlasmid II KS (Stratagene, La Jolla, CA), which were immobilized to Hybond filters using a slot blot apparatus. Hybridization was performed at 65 °C for 72 h. The filters were washed and autoradiographed. In addition, the filters were exposed to a bio-imaging plate and quantified by a bio-imaging analyzer (BAS2000, Fuji Photo Film, Tokyo, Japan).

Plasmid Constructs and Labeling of RNA—NaPi-2 RNA was transcribed from pSPORT, which contained the full-length NaPi-2 cDNA (a gift from J. Biber) (Fig. 1A). The plasmid was linearized using different restriction enzymes and transcripts spanning different parts of the template were generated using T7 RNA polymerase. The full-length NaPi-2 transcript was prepared by linearizing the plasmid with NotI; NaPi-2 RNA without most of the 3′-UTR was transcribed after linearization with NcoI, RNA for the 5′-UTR with BspHI (Fig. 1A). For the NaPi-2 3′-UTR RNA, the NaPi-2 3′-UTR cDNA was recloned by inserting the SmaI-NotI fragment of the full-length cDNA into Bluescript II KS (Stratagene, La Jolla, CA) (Fig. 1A). This fragment spans the region of the NaPi-2 cDNA from 3′-UTR to 5′-UTR. A clone in which the 5′ of the cDNA insert was adjacent to the T3 promoter was used for sense RNA synthesis after linearizing the plasmid with NotI (Fig. 1B). The full-length RNA for rat p21 in Bluescript KS (gift of B. Vogelstein) was transcribed with T3 RNA polymerase after linearization with Sall. Human p27 in Bluescript KS (gift of K. Polyak) was similarly transcribed after linearization with AccI. Rat cyclin D1 (gift of S. Bianchi) in pCRE 27 was transcribed with SP6 RNA polymerase after linearization with Ndel. Transcription of the PTH cDNA was performed as described previously (22).

Radiolabeled RNA probes for UV cross-linking were prepared from linearized templates using the appropriate RNA polymerase in a transcription reaction containing 1 μg DNA, 0.5 μM each ATP, CTP, GTP, 8 μM UTP, 2 μM BrUTP or UTP, 500 units/ml RNase inhibitor (Promega, Madison, WI), and [32P] UTP (800 Ci/mmol, 20 mCi/ml). Samples were incubated at 37 °C for 1 h, purified on Sephadex G-50 columns, and aliquots taken for scintillation counting. The specific activity of the RNA probe (0.5–1.0 × 106 cpm) was incubated with different amounts (15–60 μg) of cytoplasmic protein extracts in a final volume of 20 μl containing 10 mM HEPES, 3 mM MgCl2, 5 mM DTT, 40 mM KCl, and 5% glycerol (binding buffer). After 30 min at room temperature, heparin was added to a final concentration of 5 mg/ml to eliminate nonspecific binding, and the samples irradiated at 2.5 J/cm2 with a UV light source of 312 nm. RNAse A-XII (Sigma) was then added for 15 min at 37 °C to a final concentration of 1 mg/ml to digest unprotected RNA. The samples were heated for 5 min at 65 °C after addition of 5 μl of Laemmli sample buffer. The samples were then loaded on a 10% SDS-polyacrylamide electrophoresis gel. RNA-protein binding was visualized by autoradiography. A molecular weight marker (Bio-Rad) was also run on the gel for size estimation of the protein-RNA bands. In some experiments, proteinase K (200 μg/ml) was added. For competition experiments, unlabeled RNA was added.

In Vitro Cell Free Degradation Assay—In vitro cell free degradation was performed essentially as described (22, 25). Radiolabeled RNA transcripts (0.3 × 106 cpm) were incubated with 20–60 μg of cytoplasmic extract and 80 units/ml RNAasin to prevent nonspecific RNA degradation, in a total volume of 40 μl at room temperature. At each time point, 6 μl were transferred to a tube containing 300 μl of TRI reagent (Molecular Research Center, Cincinnati, OH) and 10 μg of tRNA and RNA extracted. Samples were run on formaldehyde-agarose gels, transferred to Hybond membranes (Amersham Pharmacia Biotech), and immediately autoradiographed. The remaining degraded transcripts at the different time points were quantified by densitometry.

In Vitro Translation Assay—A RNA transcript (1 μg) for the full-length NaPi-2 mRNA (2464 nt) or for 1043 nt were translated using a rabbit reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer’s instructions using [35S]methionine (1000 Ci/mmol, Amersham Pharmacia Biotech). Translation was conducted in the presence of proteins (30 μg). The translation product was analyzed on SDS-PAGE and autoradiographed. In some experiments, tritium amounts (200,000 cpm, equivalent to 0.2 ng) of the transcript were added to the translation reaction for estimation of RNA recovery. At the end of the experiment, RNA was extracted from a sample of the translation reac-
Renal NaPi Cotransporter-2 mRNA Stability and Translation

**RESULTS**

**Hypophosphatemia Increases NaPi-2 mRNA Levels Post-transcriptionally**—Dietary phosphate restriction is associated with up-regulation of NaPi-2 mRNA and protein. To determine if the increase in NaPi-2 mRNA levels by hypophosphatemia in vivo was transcriptional or post-transcriptional, nuclear transcript run-on experiments were performed. Weanling rats were fed a low phosphate (0.02%) or a normal phosphate (0.3%) diet. The low phosphate diet, given for 14 days, led to hypophosphatemia, hypercalcemia and increased serum 1,25-(OH)2D3 with up-regulation of NaPi-2 mRNA and protein. To determine if the increase in NaPi-2 mRNA levels by hypophosphatemia in vivo was transcriptional or post-transcriptional, nuclear transcript run-on experiments were performed. Weanling rats were fed a low phosphate (0.02%) or a normal phosphate (0.3%) diet. The low phosphate diet, given for 14 days, led to hypophosphatemia, hypercalcemia and increased serum 1,25-(OH)2D3 with up-regulation of NaPi-2 mRNA and protein (10). Nuclear transcript run-on experiments showed that the transcription of NaPi-2 and of other control genes expressed in the kidney were the same in nuclei from control and hypophosphatemic rats (Fig. 2). This result is representative of three repeat experiments. These results indicate that the effect of hypophosphatemia to increase NaPi-2 mRNA and protein levels is post-transcriptional. We then performed further studies to determine a mechanism for the in vivo effect.

**Cytosolic Proteins from Hypophosphatemic Rats Stabilize the NaPi-2 mRNA Transcript in an in Vitro Degradation Assay**—To determine a role for renal cytosolic proteins in the post-transcriptional regulation of NaPi-2 by hypophosphatemia, NaPi-2 mRNA stability was studied in an in vitro degradation assay. The labeled full-length NaPi-2 transcript was incubated with kidney cortex cytosolic proteins. At timed intervals, samples were taken and the RNA extracted and run on a formaldehyde gel to determine the level of intact radiolabeled transcript remaining. With kidney proteins from control rats, the full-length transcript was rapidly degraded at 10 min and, in contrast, with kidney proteins of the low phosphate rats, the RNA was still stable after 60 min (Fig. 3A). When RNA probes of 1043 nt (Fig. 3B) and a probe of 2065 nt (data not shown), which did not include the 3′-UTR, were incubated

with either control or low phosphate renal proteins, there was no difference in RNA degradation (Fig. 3B). However, when a transcript for the isolated 3′-UTR of NaPi-2 RNA was used in the degradation assay, the 3′-UTR transcript was stabilized by renal proteins from hypophosphatemic rats (Fig. 3C) similar to the full-length NaPi-2 transcript. This indicates that the 3′-UTR is necessary for the stabilization by low phosphate proteins. When no protein was added, there was no degradation of the transcript (Fig. 3, A and B) demonstrating that the degrading factors are in the protein extract. Quantification of the results for the degradation of either a full-length or a 1043-nt RNA are shown graphically in Fig. 3D. These results show that in this in vitro degradation assay proteins from kidneys of hypophosphatemic rats stabilized the NaPi-2 mRNA, in correlation with the increase in mRNA levels and stability found in vivo. The degradation of the probe of 1043 nt by hypophosphatemic proteins was the same as the degradation of this probe or the full-length probe by control proteins. This indicates that the 3′-UTR is necessary for hypophosphatemic renal

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**Figure 2.** NaPi-2 run-on transcription rates are the same for the nuclei of control and hypophosphatemic kidneys. Nuclear transcript run-ons for PTH receptor (PTH-R), NaPi-2, the calcium sensing receptor (CaSR), Bluestrip KS plasmid, and actin, for rats fed a control or low phosphate (Low P) diet for 14 days after weaning.

**Figure 3.** Renal cytosolic proteins from hypophosphatemic rats increase NaPi-2 RNA stability in an in vitro degradation assay. A, gel electrophoresis of full-length labeled NaPi-2 transcript incubated with cytosolic proteins from rats fed control or a low phosphate diet (low P) for different time periods. B, a 1043-nt transcript incubated with the same proteins and analyzed as in A. C, degradation of a transcript for the 3′-UTR with cytosolic proteins from control and low phosphate rats. D, time-response curves of in vitro degradation assays for the full-length NaPi-2 RNA after incubation with renal cytosolic proteins of control (●) and low phosphate (○), and the 1043-nt RNA with control (●) and low phosphate (○) proteins. Each point represents the mean ± S.E. of five different experiments. The NaPi-2 transcript is degraded very rapidly by proteins from control rats or when the 3′-UTR is not present. When the 3′-UTR is intact, hypophosphatemic proteins stabilize the transcript. This indicates that the 3′-UTR is necessary for the stabilization of NaPi-2 by low phosphate renal proteins.
proteins to stabilize NaPi-2 mRNA, and that without the 3'-UTR it is not stabilized.

Protein-RNA Binding to the NaPi-2 5'-UTR mRNA Is Increased by Hypophosphatemia—Post-transcriptional regulation is often mediated by proteins, which interact with sequences in the mRNA. To study whether protein RNA binding is involved in the post-transcriptional regulation of NaPi-2, after a low phosphate diet, we performed protein-RNA binding assays. A radiolabeled riboprobe for the NaPi-2 cDNA was prepared, incubated with rat kidney cortex proteins, cross-linked by UV light, digested with RNase A, and run on a polyacrylamide gel. In these UV cross-linking assays, specific protein-RNA bands were present which were not seen when no protein was added to the reaction mix (Fig. 4A) or when the samples were treated with proteinase K (data not shown), indicating that protein binding had protected the radiolabeled NaPi-2 mRNA sequences. The use of transcripts spanning different lengths of the NaPi-2 cDNA enabled us to determine which region of the NaPi-2 mRNA bound the cytoplasmic proteins. A radiolabeled RNA probe representing only the 5'-UTR of the NaPi-2 mRNA showed 2 prominent protein-RNA bands of approximately 40 and 55 kDa and a faint band at 35 kDa. This binding was increased when the RNA was incubated with renal proteins from rats fed a low phosphate diet (Fig. 4A). A transcript for part (1043 nt) of the NaPi-2 mRNA spanning the 5'-UTR and part of the coding region of the mRNA and also a probe for the full-length mRNA, both bound cytoplasmic proteins with the same pattern consisting of two major bands at 35 and 40 kDa (Fig. 4A). The 40-kDa protein RNA band was present with all three probes and was increased by low phosphate. The longer transcripts (1043 nt and the full-length) showed an intense band at 35 kDa which was less intense with the 5'-UTR (Fig. 4A) which may be due to different secondary structures in the RNA. These results indicate that the 5'-UTR was the region necessary for protein binding. Hypophosphatemic renal protein extracts showed increased binding to full-length NaPi-2 mRNA as compared with controls (Fig. 4A). This increase in binding of kidney proteins of hypophosphatemic rats was also observed with the shorter RNA probes spanning 1043 nt or just the 5'-UTR of the NaPi-2 mRNA (Fig. 4A).

A probe for the 3'-UTR of the NaPi-2 mRNA showed no specific binding of proteins to the RNA (data not shown), indicating that the isolated 3'-UTR did not bind renal proteins. It is noteworthy that the 3'-UTR was necessary for the effect of hypophosphatemia on the in vitro degradation of the NaPi-2 transcript (Fig. 3), but in its isolated form it did not show binding.

The same binding pattern to the NaPi-2 mRNA full-length and 5'-UTR was also demonstrated by UV cross-linking in proteins from parathyroid and liver (Fig. 4B). However, proteins from the parathyroid and liver of the same hypophosphatemic rats bound NaPi-2 mRNA at the same level as controls, indicating that the increased binding of hypophosphatemia was unique to the kidney (Fig. 4B).

Competition experiments with excess unlabeled NaPi-2 mRNA indicated the specificity of the binding of proteins to the NaPi-2 mRNA. Excess unlabeled RNA representing the first 1043 nt of NaPi-2 mRNA or its 5'-UTR competed for binding of the full-length NaPi-2 mRNA probe, confirming that the 5'-UTR was the protein binding site (Fig. 5A). Other mRNA transcripts of cDNAs such as PTH and the cell cycle inhibitors p21 and cyclin D1 (data not shown) also competed for binding to the NaPi-2 mRNA, indicating that the probes which bind the NaPi-2 mRNA may also recognize sequences in these mRNAs. In addition, PTH mRNA 5'-UTR bound renal cytoplasmic proteins (data not shown). It is of interest that the translation in vitro of all these mRNAs is regulated by renal hypophosphatemic proteins (see later).

The binding of proteins to the NaPi-2 mRNA was also demonstrated by Northwestern blots (Fig. 6). This method determines the size of the proteins that bind NaPi-2 mRNA without the bound radiolabeled RNA sequences that are present in the UV cross-linking assay. Kidney cortex proteins from control and rats fed a low phosphate diet were run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes which were hybridized with a RNA probe for NaPi-2 mRNA. The binding was increased with renal hypophosphatemic proteins (Fig. 6) but not with hepatic hypophosphatemic proteins (data not shown). These proteins were similar in size to those demonstrated by UV cross-linking. It is noteworthy that, in some experiments, an additional band of ~55 kDa was present (Figs. 4A and 5), while in other instances this larger band was very faint (Fig. 4A).

To determine a role for the differences in NaPi-2 5'-UTR
RNA-protein binding of renal proteins from hypophosphatemic rats, we analyzed their effect on NaPi-2 RNA translation.

Increased Incorporation of $[^{35}S]$methionine into NaPi-2 Protein in the Kidneys of Hypophosphatemic Rats—To demonstrate that hypophosphatemia led to an increase in NaPi-2 protein synthesis, we compared the \textit{in vivo} rates of NaPi-2 synthesis. Rats fed a normal or a low phosphate diet were injected intraperitoneally with L-$[^{35}S]$methionine. After 15 min, the kidneys were removed and kidney cortex proteins extracts prepared. Incorporation of radioactivity into renal protein was estimated by precipitation with trichloroacetic acid. NaPi-2 was isolated from the extracts by antibody precipitation. Equal amounts of radioactivity were added to an excess of L-[35S]methionine was injected intraperitoneally to control rats (N) and rats fed a low phosphate (P) diet, and after 20 min renal cortical proteins were extracted. Equal amounts of radioactivity were immunoprecipitated with NaPi-2 antibody and run on a SDS-PAGE (IP). In addition, a sample before immunoprecipitation from control and low phosphate proteins was run on a SDS-PAGE (Total protein). The gels were autoradiographed. There was an increase in labeled NaPi-2 (arrow) in low phosphate kidneys but no generalized increase in protein synthesis.

Cytosolic Proteins from Hypophosphatemic Rats Increase NaPi-2 mRNA Translation in an \textit{in vitro} Translation Assay—A functional role for the increased binding of kidney proteins to the NaPi-2 5′-UTR mRNA was provided by an \textit{in vitro} translation assay. Kidney proteins from control and hypophosphatemic rats were added with NaPi-2 mRNA transcripts to an \textit{in vitro} rabbit reticulocyte lysate translation system in the presence of $[^{35}S]$methionine. At the end of the reaction, the $[^{35}S]$labeled translation products were run on a SDS-polyacrylamide gel and the labeled NaPi-2 protein synthesized was visualized by autoradiography. There was a marked increase in translation of NaPi-2 mRNA in the presence of proteins from kidneys of rats fed the low phosphate diet (Fig. 8A). This increase was evident when the full-length NaPi-2 transcript and also when a transcript of 1043 nucleotides were used for translation (Fig. 8A). The translation of other mRNAs such as...
addition to the increase in translation of these RNAs, they all competed for binding of proteins to the NaPi-2 mRNA (Fig. 5B).

To determine whether RNA capping affected the increased translation in the presence of renal proteins from low phosphate rats, we compared the translation of both capped and uncapped transcripts for NaPi-2 and cyclin D1. The in vitro translation of both the capped and uncapped transcripts was increased with low phosphate proteins, indicating that capping had no additional effect in this assay (data not shown).

The increase in in vitro translation with hypophosphatemic renal proteins may have been due to differences in degradation of the RNA transcripts in the presence of the proteins (Fig. 3). To exclude this possibility, we added radiolabeled NaPi-2 transcript in trace amounts, together with the unlabeled NaPi-2 transcript and renal proteins to the reticulocyte assay. At the end of the experiment, RNA was extracted from a part of the reaction and run on a gel and the amount of labeled NaPi-2 transcript was visualized by autoradiography. The result showed that the same amount of transcript was present at the end of the translation assay in the presence of normal and low phosphate renal proteins (data not shown). This indicated that there was an increase in in vitro translation in this assay and not an effect on RNA degradation under the conditions of this experiment. In the degradation assay (Fig. 3), there was a decrease in NaPi-2 RNA after incubation with normal phosphate renal proteins; this is because the protein preparation for the degradation assay is designed to retain its catalytic functions.

Addition of proteins from other tissues such as liver (Fig. 8C) and parathyroid (data not shown) of these same rats did not show any difference in translation levels, indicating that the effect was specific for proteins from kidneys. The binding of hypophosphatemic proteins from these other tissues to NaPi-2 RNA was also not increased (Fig. 4B). The increased translation in vitro of NaPi-2 mRNA and other mRNAs by renal hypophosphatemic proteins correlated with the increased binding of these proteins to the NaPi-2 mRNA 5′-UTR. These effects were specific for renal proteins and not found with hepatic proteins. The results suggest that the increased binding has a functional role in the increased synthesis of NaPi-2 protein.

**DISCUSSION**

The renal adaptation to hypophosphatemia involves an increase in the proximal tubular reabsorption of phosphate that is mediated by NaPi-2 (3, 13). The increase in NaPi-2 activity involves an increase in the levels of NaPi-2 mRNA, NaPi-2 protein, and translation of the protein to the luminal membrane. In opossum kidney cells, it has been shown that a low phosphate increases NaPi-2 mRNA levels post-transcriptionally (17, 18). To understand how a low phosphate regulates cellular processes that increases NaPi-2 in vivo, we have used a model of dietary induced chronic hypophosphatemia. We show in vivo that the increase in the mRNA levels is posttranscriptional and have performed experiments to elucidate the mechanisms that contribute to this effect. An in vitro degradation assay showed that the proteins from the kidneys of hypophosphatemic rats, when incubated with a NaPi-2 transcript, led to a more stable transcript, which is dependent upon the presence of the 3′-UTR.

The parathyroid is another organ that responds to phosphate (21, 28–30). The parathyroid has a different NaPi cotransporter named PiT-1, which is homologous to the type III NaPi cotransporters, and is increased in rats fed a low phosphate diet (31). Interestingly, hypophosphatemia decreases PTH mRNA levels post-transcriptionally, and this is also mediated by cytosolic proteins interacting with the 3′-UTR of the PTH mRNA (22). In the parathyroid this interaction results in a rapid degradation of the PTH transcript. It is intriguing that the effect of phosphate in these two organs and on the different mRNAs is post-transcriptional and mediated by protein-RNA interactions at the 3′-UTR.

UV cross-linking gels showed no binding of renal proteins to an isolated NaPi-2 RNA 3′-UTR. However, the 3′-UTR was essential for stabilization of the NaPi-2 transcript by hypophosphatemic renal proteins. Moreover, the isolated 3′-UTR was stabilized by low phosphate renal proteins in the in vitro degradation assay. This suggests that the inhibition of degradation with the low phosphate proteins is not due to proteins binding to the 3′-UTR and protecting it from degradation, but rather to an effect on the degrading proteins (Fig. 9). However, our results do not exclude the possibility that in the full-length NaPi-2 RNA, proteins may bind to sequences other than the 3′-UTR and contribute to RNA stabilization by low phosphate.

At the 5′-UTR, hypophosphatemia led to an increased binding of renal cytosolic proteins as demonstrated by both UV cross-linking and Northwestern gels. The binding studies showed that hypophosphatemia increased binding to the NaPi-2 RNA 5′-UTR, only with renal cytosolic proteins, and not with proteins from other organs, such as the liver and parathyroid that also express these proteins. A number of other mRNAs tested competed for binding of renal proteins to the NaPi-2 5′-UTR by UV cross-linking. This suggests that other mRNAs have sites that recognize the same proteins, which are regulated by hypophosphatemia in the kidney. The binding of proteins to the 5′-UTR often indicates a translational role. One possibility is that these proteins are factors that are involved in the initiation of RNA translation.

To measure NaPi-2 synthesis, [35S]methionine was injected into control and hypophosphatemic rats and the renal proteins synthesized analyzed. There was more [35S]methionine incorporation into NaPi-2 in the hypophosphatemic rat kidneys. This effect in vivo was specific for NaPi-2 protein, and there was no general increase in [35S]methionine incorporation into other renal proteins, suggesting that there was more NaPi-2...
synthesized. A short labeling time was used in this *in vivo* experiment, and therefore the amount of $^{35}$S-labeled NaPi-2 protein detected represented newly synthesized NaPi-2. However, in this *in vivo* experiment, we were unable to control the ratio of $^{35}$Smethionine to unlabeled methionine from intracellular proteolysis, which may dilute the labeled amino acid. The effect of hypophosphatemia on NaPi-2 translation was therefore studied using an *in vitro* translation assay. In the presence of hypophosphatemic cytosolic proteins, there was an increase in NaPi-2 translation compared with controls. There was increased *in vitro* translation also with a shorter transcript that did not include the 3' -UTR and part of the coding region, indicating that these excluded regions were not important to this regulation. Proteins from other tissues of these same rats showed no effect on NaPi-2 translation. Of interest, the translation of a number of other transcripts was also increased, indicating that the hypophosphatemic proteins *in vitro* affected other mRNAs as well. The increase in translation correlates with the ability of these mRNAs to compete for binding of kidney protein to NaPi-2 mRNA. The increased translation of all these mRNAs by hypophosphatoproteins was specific to the kidney. These observations suggest that hypophosphatemia enhances the renal translational machinery and this may affect other mRNAs in addition to NaPi-2. However, *in vivo*, the incorporation of $^{35}$Smethionine did not show a generalized increase in translation with hypophosphatopic proteins but only an increase in NaPi-2 protein (Fig. 7). Our results do not exclude the possibility that the translation of some specific proteins were increased. It is known that the activity of other renal transporters, such as the sodium-dependent glucose and sulfate transporters, are not increased by hypophosphatemia (32–34). This suggests that, in the kidney *in vivo*, there is a mechanism that restricts the increase in translational activity after hypophosphatemia to NaPi-2.

Hypophosphatemia, therefore, increases NaPi-2 translation and the binding of renal cytosolic proteins to NaPi-2 5' -UTR, effects that are specific to the kidney. In addition, hypophosphatemia increases the NaPi-2 mRNA stability by an effect on its 3' -UTR. These mechanisms are of relevance to the understanding of both the post-transcriptional increase in NaPi-2 mRNA and the possible increase in translation of NaPi-2 protein due to hypophosphatemia. Further understanding of these mechanisms will involve characterization of the binding proteins and definition of the RNA sequences on the NaPi-2 mRNA with which they interact.

Regulation of transcript stability and translation by RNA-protein interaction is a well defined paradigm (35). RNA stability is determined by stabilizing and degrading factors which act on specific sequences in the mRNA, often in the 3' -UTR (36). Translation initiation is generally the rate-limiting step in the overall process of translation and is influenced by elements in the 5' -UTR of the mRNA (37). An increasing number of trans-acting factors, mainly proteins associated with mRNAs, are now being recognized as modulators of translation. Translation control can be very specific, aimed at translation of only part of the mRNAs in the cell or even a single one, and it can effect a wider population of mRNAs. For NaPi-2, we show that hypophosphatemia increases its RNA stability and translation. We propose a model where a low phosphate induces a change in renal cytosolic proteins, which result in less degradation (Fig. 9). This could be due to an inhibitory factor interacting with a ribonuclease rather than with the 3' -UTR, because there was no binding to the isolated 3' -UTR by UV cross-linking. Protein-RNA interactions at the 5' -UTR regulate NaPi-2 translation. This *in vivo* model can now be used to identify protein-RNA interactions that regulate NaPi-2 gene expression, which is central to renal phosphate homeostasis.

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