Parathyroid hormone-related protein (PTHrP) is a secreted protein that acts as an autocrine and paracrine mediator of cell proliferation and differentiation. In addition to its biological activity that is mediated through signal transduction cascades, there is evidence for an intracellular role for PTHrP in cell cycle progression and apoptosis. These effects are mediated through a mid-region nuclear targeting sequence (NTS) that localizes PTHrP to the region of the nucleolus where ribonucleoprotein complexes form in vivo. In this work, we show that endogenous, transfected, and in vitro translated PTHrP proteins bind homopolymeric and total cellular RNAs at salt concentrations up to 1 M. A peptide representing the PTHrP NTS was effective in competing with the wild-type protein for RNA binding, whereas a similar peptide representing the nucleolin NTS was not. Site-directed mutagenesis revealed that the binding of PTHrP to RNA was direct and dependent on preservation of a core GXXKXXK motif, embedded in the PTHrP NTS, which is shared with other RNA-binding proteins. The current observations are the first to document RNA binding by a secreted cellular protein and predict a role for PTHrP in regulating RNA metabolism that may be related to its localization in the nucleolus of cells in vivo.

Parathyroid hormone-related protein (PTHrP) is a secreted protein that is both structurally and functionally related to PTH, the major regulator of calcium homeostasis (1, 2). As a consequence of sequence homology in amino acids 1–34, PTH and PTHrP bind to a common G-protein-coupled receptor in bone and kidney through which they elicit a spectrum of biological activity related to calcium and phosphate homeostasis (3). In addition to these effects elicited through signal transduction cascades, we (4, 5) and others (6, 7) have shown that some of the biological activity of PTHrP is mediated through amino acids 87–107, which constitute a nuclear/nucleolar targeting sequence (NTS). The PTHrP NTS bears some similarity to both the lysine-rich bipartite sequences seen in proteins such as nucleolin (8) and the arginine-rich sequence in the retroviral protein Tat (9) that mediates binding of the protein to RNA as well as nucleolar targeting. The PTHrP NTS also contains, at its amino terminus, a lysine-rich motif similar to the consensus core that has been identified in numerous double-stranded RNA-binding proteins (10).

Nucleoli are the sites within the nucleus that are involved primarily in the transcription and processing of ribosomal RNA and its assembly into ribonucleoprotein complexes prior to export into the cytoplasm (11). As such, they are prominent in interphase cells undergoing rapid protein synthesis and disappear as distinct entities in mitotic or metabolically inactive cells. Morphological analysis by electron microscopic resolution defines the nucleolus as a membrane-free organelle containing regions of varying electron density (12). In previous work, we localized PTHrP, by immunoelectron microscopy, to the dense fibrillar component of nucleoli in tissue sections from fetal rat bone (4). This region represents complexes of newly transcribed 45 S rRNA and protein that are subsequently processed and assembled into ribosomes. The complex series of events involved in ribosome biogenesis depends on the presence of numerous nucleolar proteins that have known RNA binding properties.

In a manner similar to protein/protein interactions and protein/DNA interactions, the specific and stable association of RNA and protein is most often mediated through recognition motifs (13). In many cases, a larger module containing several repeats of shorter motifs is required for efficient interaction, whereas in other instances, a self-contained motif appears to be sufficient. Perhaps the best characterized and most widely recognized RNA-binding proteins are those that contain multiple copies of either ribonucleoprotein (RNP) (14) or K homology (KH) domains (15). These modules stretch over 80–100 amino acids and are often found in conjunction with RGG boxes (13). Other proteins that have been loosely categorized on the basis of structural homology and potential biological function are those such as Tat that contain an arginine-rich motif. Yet another large (>300) group of loosely categorized proteins, to which TRBP (TAR-binding protein) (16), GCN2 (17), and PKR (18) belong, are those that bind to double-stranded RNA. These proteins contain one or more copies of a double-stranded RNA (dsRNA)-binding motif with a lysine-rich core.

To extend our previous observations toward defining the role played by PTHrP in the region of the nucleolus engaged in rRNA synthesis and processing, we investigated the possibility that PTHrP binds RNA. We now demonstrate that both endogenous and transfected PTHrP proteins bind poly(G) homopolymeric RNA, GC-rich double-stranded RNA, and total cellular RNA. The interaction is of high relative affinity and is depend-
ent on the presence of a core lysine motif shared with other RNA-binding proteins.

**MATERIALS AND METHODS**

**Plasmids and Modifications**—The previously described plasmids pPTHrP3/3 (rat PTHrP) and p387–107PTHrP/3 (3) were modified to include an in-frame Myc tag. Oligonucleotides myctag 3 (5′-cggaattctgcgtgataggaactaggtgaagggagag) and myctag 4 (5′-cgctgagtcgctcgcctgctggctagcc-3′) were used to generate a polymerase chain reaction encoding the Myc epitope flanked by a 5′-EcoRI site and a 3′-XhoI site. After digestion with EcoRI and XhoI, the fragment was ligated into the pcDNA3 expression vector (Invitrogen) to generate pcDNA3myc. Oligonucleotides myctag 1 (5′-ggattctggagtcgctcgcctgctggctagcc-3′) and myctag 2 (5′-ggtaatctgcgtgataggaactaggtgaagggagag) were used to generate PTHrP DNA fragments with a 5′- BamHI site and a 3′-EcoRI site using pPTHrP3/3 and p387–107PTHrP/3 as templates. The fragments were digested with BamHI and EcoRI and ligated into the BamHI/EcoRI site of pcDNA3myc. The resulting plasmids, PTHrPmyc and PTHrPmyc-NTS, were verified by DNA sequencing. Mutations were introduced into the target plasmid pPTHrP3/3 using the Chameleon mutagenesis kit (Stratagene). The following series of mutant cDNAs was generated with selectively altered residues within one of the three RNA-binding motifs in the PTHrP NTS: M1, 8GEEKKK12 to 8GEEKKK12, M2, 96KRRQ100 to 96KGGEL100, and M3, 106KRRQ106 to 106KHER106. The resulting cDNAs, M1PTHrP, M2PTHrP, and M3PTHrP, were modified to include an N-terminal Myc tag as described above. Human FMR1 was used as a positive control to demonstrate binding to ribosomal RNA proteins (19).

**Cell Culture and Transient Transfections**—All cell culture reagents were purchased from Life Technologies, Inc., and all plasticware (Falcon) was from Becton Dickinson Labware (Lincoln Park, NJ). The PTr cell line (a kind gift of G. N. Hendy, Calcium Research Laboratory, Montreal, Canada) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated calf serum and antibiotics/antimycotic. COS-1 cells were maintained and transfected as described previously (20, 21) using polyethylenimine (PEI) (Life Technologies, Inc.) according to the manufacturer’s instructions. 1 μg of plasmid DNA was added to the rabbit reticulocyte lysate containing T7 RNA polymerase and 500 μM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 15 μg/ml aprotinin. Cell lysates were cleared of debris by centrifugation prior to RNA binding assays.

**In Vitro Transcription and Translation**—Transcription and translation were performed in vitro using pPTHrP3/3 or p387–107PTHrP/3 as a DNA template with the TNT T7 coupled rabbit reticulocyte lysate system (Promega) according to the manufacturer’s instructions. 1 μg of plasmid DNA was added to the rabbit reticulocyte lysate containing T7 RNA polymerase, 75 μM poly(U)/500 μM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 15 μg/ml aprotinin. Bound proteins were eluted from the precipitated proteins by denaturing in formamide buffer at 95 °C for 5 min, followed by centrifugation and washed three times in lysis buffer prior to resuspension in 100 μl of Laemmli sample buffer. Bound proteins (data not shown). To determine whether the PTHrP NTS is involved in the binding of RNAs, eluted from the precipitated proteins by denaturing in formamide buffer at 70 °C for 5 min, were subjected to formaldehyde-agarose electrophoresis and transferred to a nitrocellulose membrane that was exposed to x-ray film overnight. An aliquot of unlabeled cellular RNA was run in the presence of ethidium bromide to identify 28 S and 18 S ribosomal bands on the membrane.

**Peptide Synthesis and Competition Binding Assay**—Peptides corresponding to amino acids 87–107 of PTHrP (PTHrP NTS) and/or amino acids 281–301 of nucleolin (nucleolin NTS) were synthesized with a carboxyl-terminal biotin tag at the Sheldon Institute for Biotechnology (McGill University, Montreal). Competition assays were performed by incubating 2 μl of in vitro translated PTHrP with poly(G)-Sepharose in the presence of the peptides at concentrations of 10−3 to 10−5 M. The bound peptides were separated on 15% SDS-polyacrylamide gels and analyzed by immunoblotting with anti-Myc antibody. For the peptide binding studies, 10−7 M biotinylated peptides were incubated either with poly(G)-Sepharose in lysis buffer containing up to 1000 μM NaCl or with poly(A)-, poly(C)-, or poly(U)-Sepharose in the presence of 250 μM NaCl. The beads were washed, and the bound peptides were eluted by heating in Laemmli sample buffer prior to separating on 20% SDS-polyacrylamide gels. The peptides were detected by immunoblot analysis with horseradish peroxidase-conjugated streptavidin (Stratagene).

**RESULTS**

In previous studies, we identified sequence homology between amino acids 87–107 of the cellular protein PTHrP and homologous sequences in the rat protein PTHrP. We first examined the possibility that transiently expressed PTHrP could associate with total cellular RNA. COS-1 cells were transfected with an expression vector encoding full-length rat PTHrP with the Myc epitope tag at the carboxyl terminus (PTHrPmyc) to facilitate identification of the expressed protein. Lysates of PTHrPmyc-transfected or mock-transfected COS-1 cells were immunoprecipitated with control (IgG) or anti-Myc antibody. The immunoprecipitated proteins were then incubated with 32P-labeled total cellular RNA and washed, and the bound RNA was quantitated by scintillation counting. A significant proportion of the radiolabeled RNA was observed to bind anti-Myc immunoprecipitates of PTHrPmyc-transfected cells, but not in mock-transfected cells or IgG immunoprecipitates (Fig. 1B). Immunoblot analysis of the counted samples was then performed, using anti-Myc antibody, to verify the equality of expression levels of the immunoprecipitated proteins (data not shown). To determine whether the PTHrP NTS is involved in such binding, we transfected COS-1 cells with an expression plasmid encoding PTHrPmyc devoid of the NTS (PTHrPmyc-NTS) and analyzed the lysates for RNA binding. De-
PTHRP and the mature protein. PTHrP protein devoid of the NTS (PTHRPmyc–NTS) was unable to associate with homopolymeric RNA (Fig. 1B). To assess the relative affinity of the PTHrPmyc/RNA interaction, we performed binding studies in the presence of a high salt concentration, which has been shown in the past to disrupt nonspecific protein/RNA interactions (24). The association between PTHrPmyc and poly(G)-Sepharose was stable and persisted at a salt concentration of 1 M (Fig. 1C). These data suggest that PTHrP is a bona fide RNA-binding protein and that the association requires all or part of the NTS.

Profile of RNAs Recovered in Binding Studies—To identify the species of RNA that bound to PTHrP immobilized on the Sepharose beads, the binding studies were repeated using total RNA harvested from 32P-labeled HeLa cells and protein expressed in COS-1 cells from cDNAs encoding wild-type PTHrPmyc, PTHrPmyc–NTS, and human FMR1myc. RNAs eluted from the protein-bound beads were then subjected to formaldehyde-agarose gel electrophoresis and autoradiography. As shown in Fig. 2A, ribosomal RNA species bound to both wild-type PTHrPmyc and human FMR1myc, but not to PTHrPmyc–NTS. Fig. 2B demonstrates immunoblot analysis of the proteins harvested from transfected COS-1 cells that were used for RNA binding.

Binding of Endogenous PTHrP to Homopolymeric RNA—To further explore the apparent specificity of the interaction between PTHrP and poly(G) homopolymeric RNA, the Trp cell line was used as a source of endogenous PTHrP. Lysates of PTr cells were incubated with different homopolymeric RNAs conjugated to Sepharose beads, and the bound proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with PTHrP-(67–86) antiserum. As was true of transfected PTHrP, endogenous PTHrP bound specifically to poly(G), but not to poly(C), poly(A), or poly(U)-Sepharose or Sepharose beads alone (Fig. 3A). The bound protein migrated with an apparent molecular mass of 27 kDa, and the association was capable of withstanding salt concentrations up to 1 M, indicating that the interaction was specific and of apparent high affinity (Fig. 3B).

Effects of Amino Acid Substitutions within the PTHrP NTS on RNA Binding—To define the minimum sequence within the NTS required for RNA binding activity, we tested several PTHrP constructs that contain mutations in this region (Fig. 4A). We have shown previously that mutation of 87GKKKK91 to 87GEEK91 (M1PTHrPmyc) effectively blocked the nuclear/nucleolar targeting function of the PTHrP NTS. Substitution of 102KKKRR106 for 102IIERG106 (M3PTHrPmyc) was only partially effective in this respect, and mutation of 96KRREQ100 to 96KGTTEL100 (M2PTHrPmyc) had little or no effect on targeting the protein to the nuclear compartment (33). To determine whether the proteins expressed from these cDNAs bound RNA, COS-1 cells were transfected with the expression vectors, and the lysates were used for homopolymeric binding studies. All of the proteins bound poly(G)-Sepharose at 250 mM salt, but considerable differences were observed at higher salt concentrations (Fig. 4B). The binding of both M1PTHrPmyc and M3PTHrPmyc to poly(G)-Sepharose was absent or severely impaired at 750 mM salt, whereas the binding of M2PTHrPmyc was unaffected at that salt concentration (Fig. 4B). These data suggest that the overall composition of the basic residues in the NTS affects its interaction with poly(G) homopolymeric RNA. We also examined the ability of the mutant proteins to associate with total cellular RNA (Fig. 4C). M2PTHrPmyc and wild-type PTHrPmyc bound radiolabeled cellular RNA with similar apparent affinities. However, the ability of M1PTHrPmyc to bind RNA was severely impaired, and that of M3PTHrPmyc

[Image 53x385 to 292x729]

**Fig. 1. Binding of PTHrP to total cellular RNA and homopolymeric RNA.** Total cellular RNA was harvested from 32P-labeled HeLa cells and incubated with protein precipitated with anti-Myc antibody or with IgG as a control. A, shown are the cpm of radiolabeled cellular RNA bound to protein precipitated with anti-Myc antibody (banded bars) or IgG (white bars) from mock-transfected or PTHrPmyc (PTHRP)-transfected COS-1 cells or from cells expressing PTHrPmyc–NTS (PTHRP–NTS). Bars represent the mean ± S.D. of triplicate wells from three different transfections. B, COS-1 cells expressing full-length PTHrPmyc or PTHrPmyc–NTS were lysed, and after removing an aliquot of total cell lysate (TCL), equal amounts of protein were incubated in the presence of 250 mM salt with Sepharose beads (Seph) conjugated to synthetic poly(A), poly(C), poly(G), or poly(U) RNA. Bound proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, which were probed with anti-Myc antibody. Only poly(G)-Sepharose (lane 5) bound a significant quantity of wild-type PTHrPmyc, the association being disrupted in the absence of the NTS (lane 7). The positions of molecular mass markers (in kilodaltons) are shown on the left. C, incubation of equivalent amounts of protein from COS-1 cells expressing wild-type PTHrPmyc with poly(G)-Sepharose in the presence of increasing salt concentrations did little to disrupt the association between PTHrPmyc and RNA.
was partially impaired (Fig. 4C). These observations were consistent with the data demonstrating compromised binding of the mutant PTHrPmyc proteins to poly(G)-Sepharose.

**PTHrP NTS Peptide Competes with in Vitro Translated PTHrP for RNA Binding**—We examined the possibility that the interaction between PTHrP and RNA was direct by incubating in vitro translated PTHrP with poly(G) RNA. Radiolabeled PTHrP was generated by incubating the full-length rat PTHrPmyc cDNA in the presence of [3H]leucine in a rabbit reticulocyte lysate. In vitro translated PTHrP bound poly(G)-Sepharose, and the binding persisted at a 1 M salt concentration (Fig. 5A), suggesting that the association between PTHrP and RNA was direct. The specificity of the association was then tested by performing competition binding studies in the presence of peptides corresponding to either the PTHrP NTS or the nucleolin NTS. Amino acids 87–107 of PTHrP and amino acids 281–301 of nucleolin are similar lysine-rich bipartite motifs that target the respective proteins to the nuclear compartment (4, 8). When in vitro translated PTHrPmyc was incubated with poly(G)-Sepharose in the presence of the PTHrP NTS peptide at a concentration of 10^{-4} M, the interaction between PTHrPmyc and RNA was effectively blocked (Fig. 5B). The nucleolin NTS peptide, on the other hand, was ineffective in this capacity even at 10^{-3} M (Fig. 5B). The high concentration of peptide required to dislodge in vitro translated PTHrPmyc from poly(G) homopolymeric RNA was a further indication that the association was specific and of apparent high affinity. These competition data suggested that the PTHrP NTS peptide might bind RNA directly. This possibility was tested by incubating biotinylated PTHrP NTS peptide with the different homopolymeric RNAs. The bound peptides were separated by SDS-polyacrylamide gel electrophoresis and detected by horseradish peroxidase-conjugated streptavidin. As was true of the full-length protein, the PTHrP NTS peptide bound specifically to poly(G)-Sepharose beads (Fig. 5C), and the binding withstood salt concentrations up to 1000 mM. The nucleolin NTS, on the other hand, was incapable of binding to any of the synthetic homopolymers. These experiments confirmed that the interaction between the PTHrP NTS and poly(G)-Sepharose was specific and direct and was not merely a function of its ionic strength.

**Binding of PTHrP to Double-stranded Nucleotide Sequences**—TRBP is one of a number of cellular proteins that associate with GC-rich double-stranded RNA, including the stem-loop structure formed by the Tat transactivating or TAR region of

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**Fig. 2.** Profile of cellular RNAs bound to transiently expressed PTHrP. Total cellular RNA harvested from 32P-labeled HeLa cells was incubated with proteins precipitated from COS-1 cells transfected with cDNAs encoding PTHrPmyc, PTHrPmyc–NTS (−NTSPThPmyc), or human FMR1myc (hFMRmyc). A, bound RNA eluted from precipitated proteins was resolved on formaldehyde-containing 1% agarose gels and transferred to nitrocellulose membranes that were exposed to x-ray film overnight. Positions of 28 S and 18 S ribosomal bands were verified by ethidium bromide staining. Labeled RNA was eluted from anti-Myc immunoprecipitates (IP) from lysates of cells expressing PTHrPmyc and human FMR1myc, but not from PTHrPmyc–NTS-expressing cells or when IgG was used in place of anti-Myc antibody. B, shown are the results from immunoblot analysis of aliquots of Sepharose beads recovered by immunoprecipitation.
dependent on the presence of a core GXX motif within PTHrP that is conserved among other RNA-binding proteins. A peptide corresponding to the PTHrP NTS can bind alone to poly(G)-Sepharose and can compete with wild-type PTHrP for binding to the homopolymer. Site-directed mutagenesis revealed that the RNA binding activity of PTHrP was direct and dependent on preservation of the core motif.

The endocrine, paracrine, and autocrine mechanisms of PTHrP action, which are mediated through transduction cascades following the interaction of secreted PTHrP with the common PTH/PTHrP cell-surface receptor, are well documented. On the other hand, little is known regarding the documented intracellular role played by PTHrP. The PTHrP NTS is situated in a highly conserved region of the protein that has also been identified as a site of endoproteolytic cleavage (25). However, the presence of an intact NTS has been shown to have biological relevance not only in protecting serum-deprived chondrocytes from apoptosis (4), but also as a requirement for PTHrP-induced mitogenesis in vascular smooth muscle cells (6). Others have shown that PTHrP is targeted to the nucleolus of cells in G1 and suggest that its subcellular distribution is cell-cycle dependent (7). As a first step toward defining the molecular basis for these intracellular actions of PTHrP, we now show that PTHrP is a sequence-specific RNA-binding protein and that the association with RNA is dependent on the presence of a core motif found in double-stranded RNA-binding proteins.

In the absence of highly specific PTHrP antibodies, we modified all of our expression constructs to include a Myc epitope tag. Using the anti-Myc antibody, two major species of PTHrP were commonly detected in immunoblot analysis of lysates prepared from PTHrPmyc-transfected COS-1 cells. They migrated with apparent molecular sizes slightly larger (due to the 11-amino acid Myc tag) than those previously detected with an antiserum raised against the amino terminus of PTHrP (4). An antiserum that was raised against synthetic PTHrP-(67–86) recognized a major species of endogenous PTHrP that bound to poly(G)-Sepharose and migrated with an apparent size similar...
to that of prepro-PTHrP. Thus, it appeared that more than one species of PTHrP could associate with homopolymeric RNA.

The strength of binding of a protein to a specific nucleotide sequence, whether it be DNA or RNA, can be assessed by performing the binding studies at high salt concentrations. If the binding is weak or requires additional sequences outside of those being tested, then the association will not withstand an elevation in salt concentration (13). In this context, binding of the KH domain proteins hnRNP K and FMR1 to RNA is mediated through a combination of KH domains and an RGG box (24, 26). Binding of in vitro translated wild-type FMR1 to poly(G)-Sepharose was severely compromised at a salt concentration of 500 mM. Binding of the prototypical hnRNP K protein to RNA, on the other hand, was resistant to 1 M salt, as was demonstrated for PTHrP in our own work. Homopolymeric RNA binding in the presence of high salt concentrations was abrogated by deletion of the RGG box or the carboxyl-terminal KH domain in FMR1 (24). Variable alterations in binding to RNA at high salt concentrations resulted from point mutations of critical Ile residues in the three KH domains in hnRNP K (26). In a similar manner, we have shown that deletion of the PTHrP NTS results in abrogation of binding to poly(G)-Sepharose and that conservative mutations within the NTS lead to variable alterations in binding to RNA at high salt concentrations. It is interesting to note that, despite their lack of structural similarities, PTHrP and FMR1 both bind 28 S and 18 S species of ribosomal RNA with an apparent equal affinity at low salt concentrations. As is true for many other RNA-binding proteins, the physiological significance of the different structural motifs and their RNA binding affinities and specificities awaits further investigation.

In the absence of the NTS, there was no specific binding to total cellular RNA or to homopolymeric RNA. However, deletion of a stretch of 20 amino acids could influence the overall structure of PTHrP, thus preventing it from making contact with its RNA target. We therefore sought to map the point of contact between PTHrP and RNA using a set of NTS mutants that had previously been generated for immunofluorescent localization of the protein. In those studies, it was shown that substitution of the amino-terminal lysines was most effective in

FIG. 5. PTHrP binding specificity confirmed using competition with NTS peptides. PTHrPmyc was transcribed and translated in vitro in the presence of [3H]leucine using a rabbit reticulocyte lysate. A, equal volumes of lysate were incubated with the different homopolymers at 250 mM salt and with poly(G)-Sepharose at salt concentrations up to 1 M. Significant binding of in vitro translated PTHrP was seen only for poly(G) and was maintained up to 1 M salt (lanes 6-9). Peptides corresponding to the PTHrP NTS or the nucleolin NTS were synthesized for use in competition binding assays using the in vitro translated PTHrPmyc. B, equal volumes of lysate were incubated with poly(G)-Sepharose in the presence of increasing concentrations of NTS peptides at a salt concentration of 250 mM. Despite their similar bipartite lysine motifs, the PTHrP NTS effectively blocked binding of PTHrPmyc at a concentration of 10⁻⁴ M (lane 7), whereas the nucleolin NTS was ineffective even at 10⁻³ M (lane 10). C, 10⁻⁷ M biotinylated PTHrP NTS peptide was incubated with the different homopolymers in the presence of 250 mM NaCl and with poly(G)-Sepharose in the presence of increasing NaCl concentrations. The nucleolin NTS peptide was incubated with poly(G)-Sepharose in buffer containing 250 mM NaCl. Bound peptides were separated on 20% SDS-polyacrylamide gels and visualized with horseradish peroxidase (HRP)-conjugated streptavidin. Distinct bands of peptide alone were observed for the PTHrP NTS (lane 1) and for the nucleolin NTS (lane 11). The PTHrP NTS bound to poly(G)-Sepharose (lanes 5 and lanes 7-10) in the presence of NaCl concentrations up to 1 M (lane 10). Seph, Sepharose; TCL, total cell lysate.

FIG. 6. Binding of PTHrP to double-stranded RNA. Proteins expressed in COS-1 cells were used for binding to synthetic double-stranded nucleotide sequences in the presence of 250 mM salt. A, in the presence of a low salt concentration, PTHrPmyc, as well as the three mutant proteins, bound poly(G) (lane 3) and poly(G)-poly(C) (lane 5) with the same apparent affinity. None of the proteins demonstrated significant binding to the corresponding deoxynucleotide sequences (lane 6). B, binding of wild-type and mutant forms of PTHrPmyc to double-stranded poly(G)-poly(C) RNA demonstrated the same pattern of affinities as seen for single-stranded poly(G). TCL, total cell lysate; Seph, Sepharose.
preventing nuclear/nucleolar targeting of PTHrP (33). More recently, others have demonstrated that proteins in which either the amino- or carboxyl-terminal lysine tract was deleted failed to stimulate proliferation when expressed in vascular smooth muscle cells, as did intact PTHrP (6). We now show that substitution of these same basic amino acids, particularly the amino-terminal stretch, compromises binding of the protein to both synthetic and total cellular RNAs. This is in keeping with the observation that PTHrP $^{87}$GXXKXXXX constituting a core motif that is also found in the functionally heterogeneous group of proteins containing dsRNA-binding domains. The general structure of the dsRNA-binding domain is αββα, and it is believed that the junction between the third β-sheet and the α-helix plays a critical role in RNA binding (10). PTHrP does not conform to these structural requirements in that it has helix-breaking prolines at positions 86 and 94 that flank the core motif and does not possess the extended dsRNA-binding domain. Despite this lack of overall homology, mutation of specific lysines within the core motif resulted in severely compromised binding of PTHrP to RNA in our own studies as well as in those of others examining binding of the dsRNA-binding domain proteins PKR (27) and GCN2 (17) to RNA. In the case of PTHrP, substitution of other lysine or arginine residues in the NTS had only a modest or no effect on binding.

Another member of the family of dsRNA-binding proteins is TRBP, which was isolated from a HeLa cell expression library on the basis of its ability to bind with high affinity to the GC-rich double-stranded stem of TAR RNA (16, 28). We have demonstrated that PTHrP binds avidly to G-rich sequences in both a single- and double-stranded stem (29). It is therefore possible that the cellular RNA avidly to G-rich sequences in both a single- and double-stranded context. It is therefore possible that the cellular RNA

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