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Synthesis and Characterization of Extended and Deleted Recombinant Analogues of Parathyroid Hormone-(1-84): Correlation of Peptide Structure with Function†

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Received May 14, 1990; Revised Manuscript Received July 13, 1990

ABSTRACT: Recombinant analogues of human parathyroid hormone [hPTH-(1-84)] were expressed in *Escherichia coli* harboring plasmids containing synthetic genes under the control of the lac promoter. The level of expression of the gene encoding the truncated analogue, hPTH-(3-84), was greater than that of the gene encoding full-length hPTH-(1-84) but less than that of the gene encoding proparathyroid hormone (hProPTH). This may be due in part to the relative efficiency of translation of the mRNA as suggested by secondary structure analysis and in part because of enhanced stability of the extended peptide. Formylmethionyl derivatives of hProPTH and of hPTH-(3-84) and underivatized hPTH-(3-84) were purified by HPLC, and their identity was confirmed by NH_2-terminal sequencing and amino acid analysis. The bioactivity of these recombinant peptides was then tested in skeletal and renal adenylate cyclase assays in vitro and in assays examining effects on plasma and urine calcium and phosphate levels and on urine cyclic AMP levels in vivo. The NH_2-terminally extended analogue fMet-hProPTH displayed 10% of the in vitro activity of hPTH-(1-84) and was a partial agonist in vivo. The peptides hPTH-(3-84) and fMet-hPTH-(3-84) were inert in vitro and were very weak in vitro antagonists when compared to the NH_2-terminal analogue bovine [$Nle^{8,18}Tyr^{34}$]PTH-(3-34)-NH_2. In vivo, hPTH-(3-84) and the bPTH-(3-34) analogue, when assayed at a 10:1 molar ratio relative to bPTH-(1-84), were each inert, and neither demonstrated antagonist activity at these concentrations. The results demonstrate a variation in expression levels of synthetic genes encoding deleted, full-length, and extended forms of hPTH-(1-84) and show that either NH_2-terminal or COOH-terminal extension of the active region of the PTH molecule may produce conformational changes which apparently alter receptor binding and reduce both agonist and antagonist activity.

Parathyroid hormone (PTH)[1] (Potts et al., 1982; Goltzman & Hendy, 1990) interacts with renal and skeletal receptors to regulate extracellular calcium and phosphate concentrations at least in part through stimulation of the adenylate cyclase system. Although the major glandular, and secreted, bioactive form of the hormone is an 84 amino acid straight-chain peptide, PTH-(1-84), the active core of the molecule is believed to reside within the NH_2-terminal sequence 1-27 (Tregear et al., 1973), and to date, studies correlating PTH structure with function have employed mainly analogues of the synthetic NH_2-terminal peptide, PTH-(1-34). The latter peptide interacts with specific, high-affinity receptors in renal and osseous tissue and appears to carry out most of the functions of the intact molecule. Nevertheless, specific binding sites for the midregion and carboxyl regions of PTH-(1-84) have been described (McKee & Murray, 1985; Demay et al., 1985), and recent studies have implicated these domains in hormonal effects on cell replication and differentiation (Schluter et al.,

† This work was supported by Grants MA-9315 and MT-5775 from the Medical Research Council of Canada. This is NRCC Publication No. 31885.

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‡ Recipient of a Fellowship Award from the MRC of Canada.
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‖ Recipient of a Scholarship Award from the MRC of Canada.

[1] Abbreviations: PTH, parathyroid hormone; h, human; b, bovine; fMet, formylmethionyl; HPLC, high-performance liquid chromatography; IPTG, isopropyl thiogalactoside.

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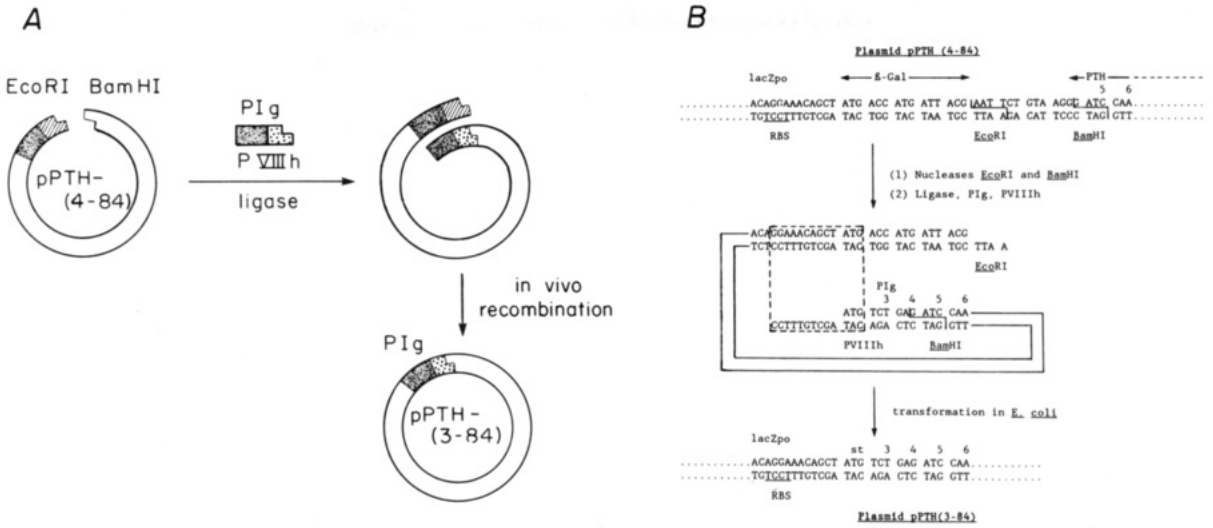

FIGURE 1: (A) General scheme for construction of *E. coli* plasmid vector pPTH-(3–84) that directs the expression of hPTH-(3–84) DNA. The heavily stippled bar represents the sequence from the ribosomal binding site to the start codon ATG of the *E. coli* β-galactosidase gene. The lightly stippled bar represents synthetic oligonucleotides encoding the amino-terminal portion of hPTH, and the hatched bar represents the NH_2 terminus of the β-galactosidase gene. The dephosphorylated *Eco*RI–*Bam*HI restriction fragment from pPTH-(4–84) was ligated with phosphorylated oligonucleotides PIg and PVIIIh as described under Experimental Procedures. In subsequent transformation of *E. coli* JM103, the homology-searching sequence (heavily stippled bar) of the PIg·PVIIIh duplex recombined intramolecularly with the lac promoter-operator to complete the circularization of the plasmid. Through this operation, the NH_2-terminal β-galactosidase sequence was specifically deleted, and the PTH-(3–84) gene was directly linked to the lac operator to form plasmid pPTH-(3–84). (B) Details of construction of plasmid pPTH-(3–84) from plasmid pPTH-(4–84) by intramolecular recombination. lacZpo is the promoter-operator region of the lacZ gene and RBS is the ribosomal binding site of the β-galactosidase gene (β-Gal). PIg and PVIIIh are synthetic oligonucleotides and make up the coding strand and complementary strand, respectively, of the ribosomal binding site, the ATG, and the nucleotides encoding amino acids 3 and 4 of hPTH. The sequences of oligonucleotides PIg and PVIIIh are 5′-ATGTCTGA-3′ and 5′-GATCTCAGACATAGCTGTTTCC-3′, respectively. The homologous sequences for intramolecular recombination between the lac operator and the oligomers PIg·PVIIIh in the plasmid intermediate are contained in the box.

1989; Murray et al., 1989). Previous studies have demonstrated the marked reduction in bioactivity which accompanies alterations of the NH_2 terminus of PTH-(1–34) and have shown that inert synthetic analogues of PTH-(1–34), truncated at the NH_2 terminus, may act as hormonal antagonists (Goltzman et al., 1975; Horiuchi et al., 1983). A single report (Born et al., 1988) has described the bioactivity of recombinant human PTH-(3–84) and has shown this to be a more potent antagonist, in an in vitro bioassay, than an analogue of PTH-(3–34). To explore these issues further, we have examined the bioactivity of extended and deleted forms of PTH-(1–84), the major circulating bioactive species of the hormone. Recombinant peptides were produced in *Escherichia coli* transformed with plasmids constructed to contain synthetic genes encoding human PTH-(1–84), the deleted form, hPTH-(3–84), and the extended form, proparathyroid hormone [hProPTH; extended at the NH_2 terminus by the hexapeptide (KSVKKR)]. Recombinant molecules were purified to homogeneity, characterized biochemically, and assessed in bioassays in vitro and in vivo.

Experimental Procedures

Materials. *E. coli* K12 strain JM103 [Δ(*lac pro*), *thi*, *str A*, *sup E*, *end A*, *sbc B*, *hsd R*, *Ftra* D36, *pro* AB, *lac* I^Q, Z M15] was used as the bacterial host. Synthetic oligonucleotides were prepared with an Applied Biosystems DNA synthesizer, Model 380B. Enzymes were purchased from Bethesda Research Laboratory [T_4 DNA ligase, calf intestinal alkaline phosphatase (CIAP), and restriction endonucleases *Eco*RI, *Bam*HI, and *Pst*I] and New England Biolabs (T_4 DNA kinase). Synthetic hPTH-(1–84) and the synthetic analogues bovine [$Nle^{8,18}Tyr^{34}$]PTH-(3–34)-NH_2, [Tyr^{34}]-bPTH-(7–34)-NH_2, and hPTH-(7–84) were purchased from Bachem Laboratories (Torrance, CA). For in vivo bioassay studies, bPTH-(1–84) code 76/572 was obtained from the National Institute of Biological Standards and Control, London, U.K. This has a biological activity of 2500 MRC units/mg.

Construction of Expression Plasmids. (*A*) *pPTH-(3–84) Expression Plasmid.* A precursor plasmid, pPTH-(4–84), was prepared via ligation of phosphorylated oligonucleotides PII, PIII, PVI, and PVII with the *Bam*HI/CIAP/*Pst*I-treated plasmid pPTH-84 [see Rabbani et al. (1988b) and Sung et al (1986a,b) for description of the oligonucleotides]. Figure 1A outlines the construction scheme of plasmid pPTH-(3–84). The precursor plasmid, pPTH-(4–84), was linearized by restriction nucleases *Eco*RI and *Bam*HI. Oligonucleotide PVIIIh (6 pmol; see Figure 1B) was phosphorylated in a solution (10 μL) containing 0.3 mM ATP, 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 5 mM DTT, and 30 units of T_4 DNA kinase at 37 °C for 1 h. After being heated at 70 °C for 6 min, oligonucleotide P1g was added. The solution was cooled to 22 °C slowly and added to a solution (4 μL) containing 75 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 12.5 mM DTT, 1.2 mM ATP, 2 units of T_4 DNA ligase, and 50 ng (0.025 pmol) of *Eco*RI/*Bam*HI-treated plasmid pPTH-(4–84). After incubation at 12 °C for 16 h, the ligation mixture was used to transform *E. coli* JM103 on YT plates (8 g of Bacto tryptone, 5 g of Bacto yeast extract, 5 g of NaCl, and 15 g of Bacto agar in 1 L) containing 100 μg of ampicillin/mL. Transformants were selected randomly for hybridization with ^{32}P-labeled probe PVIIIh (washed at 55 °C). Among 172 candidates, 18 transformants hybridized to the probe. The construction of the purified plasmid in the region of the ribosomal binding site was confirmed by nucleotide sequencing.

(*B*) *pPTH-(−6→+84) Expression Plasmid.* (*i*) *Construction of Plasmid pProPTH-1.* Figure 2 outlines the construction of the *E. coli* plasmid vector encoding hProPTH. Phosphorylated oligonucleotide PRO-1 (80-fold molar excess)

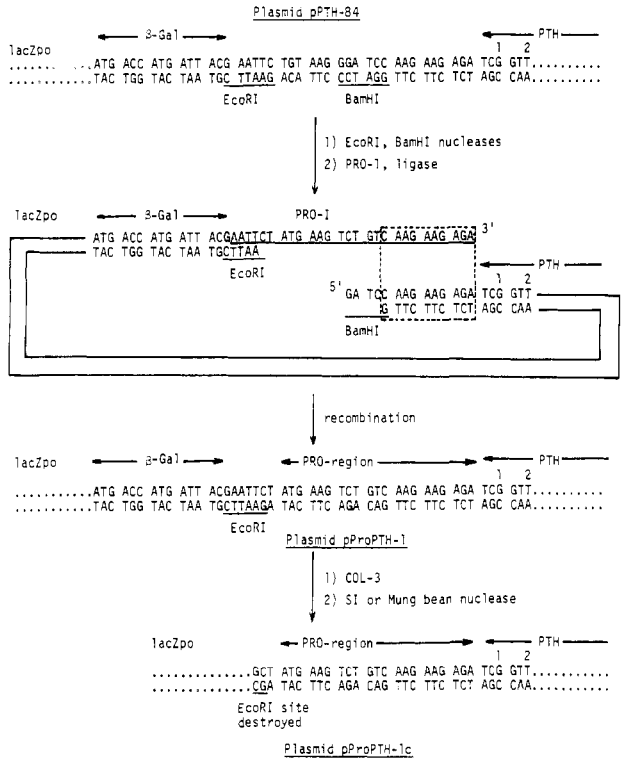

FIGURE 2: Scheme for construction of *E. coli* plasmid vector pPTH-(−6→+84) that directs the expression of hProPTH DNA. lacZpo is the promoter-operator region of the lacZ gene, and β-Gal is the NH_2 terminus of the β-galactosidase sequence. The homologous sequences for intramolecular recombination between oligomer PRO-1 and plasmid pPTH-84 are contained in the box. Dephosphorylated *Eco*RI-digested pProPTH-1 was ligated with phosphorylated oligomer COL-3 as described under Experimental Procedures, and in subsequent transformation of *E. coli* JM103, the NH_2-terminal sequence of β-galactosidase was deleted by homologous recombination. The *Eco*RI site was destroyed by S1 nuclease digestion and blunt-end ligation to yield plasmid pPTH-(−6→+84) (designated here as pProPTH-1c). The sequences of oligonucleotides PRO-1 and COL-3 are 5′-AATTCTATGAAGTCTGTCAAGAAGAGA-3′ and 5′-TTGTTAAAGTGTGTCCTTTGTCTTAA-3′, respectively.

was ligated with *Bam*HI/*Eco*RI/CIAP-treated plasmid pPTH-84 (Sung et al., 1986a,b). After transformation, positive colonies were identified by hybridization with ^{32}P-labeled probe PRO-1 [see Sung et al. (1989) for description of this oligonucleotide]. The new plasmid pProPTH-1 was present in 34% of all transformants (Sung et al., 1989). The pro region was confirmed by nucleotide sequencing.

(*ii*) *Construction of Plasmid pProPTH-1B.* Phosphorylated cross-over linker COL-3 [see Sung and Zabab (1987) for description of oligonucleotide] (20-molar excess) was ligated with *Eco*RI/CIAP-treated plasmid pProPTH-1. After transformation and hybridization with ^{32}P-labeled probe COL-3, 14% of the transformants were identified as containing pProPTH-1B. This was confirmed by nucleotide sequencing.

(*iii*) *Construction of Plasmid pProPTH-1C.* Plasmid pProPTH-1B was linearized with endonuclease *Eco*RI and incubated with S1 nuclease at 37 °C for 10 min. After blunt-end ligation and transformation, 19% of all colonies contained plasmid pProPTH-1C as indicated by the lack of hybridization with ^{32}P-labeled probe COL-3. This was confirmed on the purified plasmid by nucleotide sequencing. Plasmid pProPTH-1C is subsequently designated in the text as plasmid pPTH-(−6→+84).

Expression in E. coli. E. coli transformants bearing either pPTH-(1–84), pPTH-(3–84), or pPTH-(−6→+84) were grown in Luria broth containing ampicillin, and hPTH gene transcription was induced with 1 mM IPTG as described previously (Rabbani et al., 1988b). Cells were harvested for radioimmunoassay before and after addition of chloramphenicol to inhibit protein synthesis, as described (Rabbani et al., 1988b).

Messenger RNA Analysis. E. coli harboring either the pPTH-(1–84), pPTH-(3–84) or pPTH-(−6→+84) plasmids were grown in Luria broth containing ampicillin, and hPTH gene transcription was induced with 1 mM IPTG as described previously (Rabbani et al., 1988b). After 7 h at 37 °C, 1-mL aliquots of cells were harvested, and RNA was isolated as described (Ivanov et al., 1987), treated with 10 μg/mL DNase I (RNase free), deproteinized by phenol extraction, and precipitated with ethanol. RNA was dissolved and denatured, and serial dilutions were dotted onto nylon filters as described (Brookman et al., 1986). Duplicate filters were hybridized under standard conditions with either a polynucleotide kinase ^{32}P-labeled oligonucleotide PVI, which is complementary to amino acids 13–28 encoded by the synthetic hPTH gene of pPTH-(1–84) (Sung et al., 1986a,b), or a ^{32}P random primer labeled 1500-bp *Taq*I–*Taq*I restriction fragment encoding the ampicillinase gene from pUC8. After autoradiography, the intensities of the hybrid images were quantitated by laser densitometry.

The 5′ 375 nucleotides, which contain the initiator AUG codon regions, of mRNAs encoding either hPTH-(1–84), hProPTH-(−6→+84), or hPTH-(3–84) were analyzed for potential secondary structures with the Zuker computer program RNA-2 (Zuker & Stiegler, 1981).

Peptide Extraction Procedure. Cells were homogenized in acetone and *n*-hexane, and the dried residue was extracted in a mixture of 1 M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl, and 1% (v/v) trifluoroacetic acid (CF_3COOH), as described previously (Rabbani et al., 1988b; Bennett et al., 1981).

HPLC. The supernatants from the acid extraction of the dried and defatted *E. coli* cells were either pumped directly onto a Prep-Pak C_{18} cartridge and step eluted with 80% acetonitrile as described (Rabbani et al., 1988b; Bennett et al., 1981) or batch extracted from Sep-Pak C_{18} cartridges as described (Bennett et al., 1981). C_{18} silica extracts were then subjected to sequential reversed-phase HPLC on C_{18} μBondapak columns eluted with gradients of acetonitrile containing either 0.1% CF_3COOH or 0.13% heptafluorobutyric acid (C_3F_7COOH) as described (Rabbani et al., 1988b). Gel filtration HPLC of purified peptides was carried out as described (Bennett et al., 1981).

Immunoassays. Radioimmunoassays for parathyroid hormone were carried out as previously described (Goltzman et al., 1984) with either guinea pig antiserum GP 26 raised against bPTH-(1–84) and [^{125}I-Tyr^{52}]hPTH-(52–84) as tracer (C assay) or a radioimmunometric assay of intact hPTH (intact assay), performed with the Allegro Kit (Nichols Institute, San Juan Capistrano, CA) (Nussbaum et al., 1987). The standard used was synthetic hPTH-(1–84).

Bioassays. In vitro adenylate cyclase assays were performed in rat osteosarcoma cells (UMR 106) and opossum kidney cells (OK) as described previously (Rabbani et al., 1988a) with an assay incubation medium consisting of minimum essential medium (MEM) buffered with Hank's salts (GIBCO, Burlington, ON), 0.2% BSA (Sigma, St. Louis, MO), and 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma). In both bioassays the standard used was synthetic hPTH-(1–84) (Bachem) which has a biological activity of 2500 MRC units/mg. In vivo bioassays were performed as described previously (Rabbani et al., 1988a) in thyroparathyroidectomized rats. Following a 1-h control urine collection, a blood

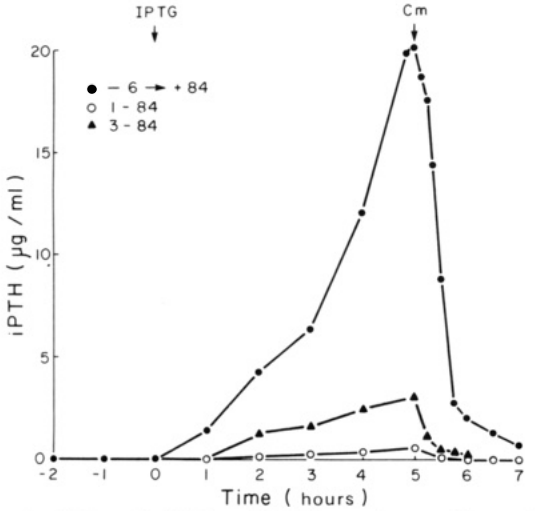

FIGURE 3: Effect of addition of IPTG to cultures of *E. coli* cells harboring either plasmid pPTH-(3–84) (▲), plasmid pPTH-(1–84) (○), or plasmid pPTH-(−6→+84) (●). The cultures were grown, aliquots taken at the times indicated, and cell extracts made, and radioimmunoassay (C assay) was carried out as described under Experimental Procedures. Chloramphenicol (Cm; 150 μg/mL) was added after 5 h to inhibit protein synthesis.

sample was taken at time zero. Peptides were then infused for 2 h, during which time urine collections were made over 30-min periods, blood samples being taken at the end of each urine collection. Biochemical analyses and data analyses were as described (Rabbani et al., 1988a).

Amino Acid Analysis and Amino Acid Sequencing. Amino acid analyses of the purified peptides were carried out with a Pico-Tag Work Station (Waters) and a Beckman System 6300 analyzer, and amino acid sequencing was performed on an Applied Biosystems (gas phase) sequencer as described previously (Rabbani et al., 1988a).

Results

Construction of Expression Plasmid hPTH-(3-84). The PTH-(3–84) gene of the newly generated expression plasmid has, like the hPTH-(1–84) expression plasmid, an upstream control sequence identical with that of a lacZ gene (Figure 1).

Construction of Expression Plasmid hPTH-(−6→+84). The cross-over linker PRO-1 (Sung et al., 1989), encoding a start codon and the pro sequence of hProPTH, was designed with a 10-base homology-searching sequence for precise integration with a synthetic hPTH gene previously cloned into the lacZ gene of plasmid pUC8 (Sung et al., 1986a,b). The resultant plasmid pProPTH-1 still maintained a residual β-Gal sequence fused with the ProPTH gene. The second cross-over linker, COL-3 (Sung & Zahab, 1987), with a 22-base homology-searching sequence targeting the ribosome-binding site of the lacZ gene, efficiently deleted the β-Gal gene residue to yield plasmid pProPTH-1B. The restored *Eco*RI site of pProPTH-1B was eliminated by linearization, S1 nuclease degradation, and blunt-end ligation. The ProPTH gene of the newly generated plasmid pProPTH-1C has an upstream control sequence identical with that of a lacZ gene (Figure 2).

Characteristics of the Expression of Immunoreactive Recombinant PTH. Figure 3 shows the effect of IPTG induction on intracellular immunoreactive hPTH in *E. coli* cells harboring expression plasmids containing either the hPTH-(1–84) gene, the hProPTH gene, or the hPTH-(3–84) gene. Five hours after addition of IPTG, the level of immunoreactive hPTH-(3–84) was 5-fold that of hPTH-(1–84) but only 12.5%

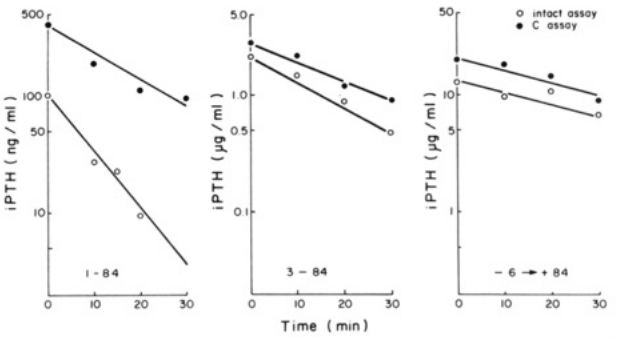

FIGURE 4: Comparison of the stability of immunoreactive PTH in *E. coli* cells containing pPTH-(1–84), pPTH-(3–84), or pPTH-(−6→+84) plasmids. Transcription of the hPTH gene was induced by addition of IPTG, and chloramphenicol was added after 5 h to inhibit protein synthesis. The clearance of immunoreactive PTH was monitored by a carboxyl-terminal specific assay (●) and an intact molecule assay (○).

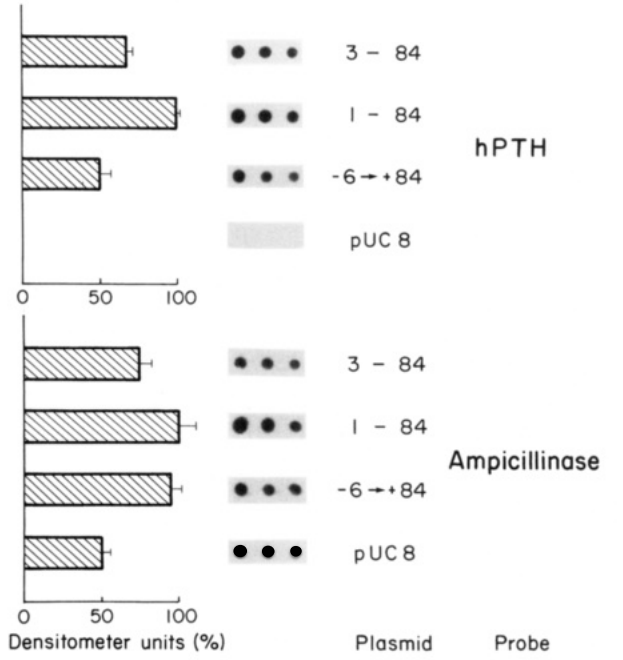

FIGURE 5: Relative hPTH and ampicillinase mRNA levels in cultures of *E. coli* cells harboring either plasmid pPTH-(3–84), plasmid pPTH-(1–84), plasmid pPTH-(−6→+84), or plasmid pUC8. Cultures were grown, and RNA was extracted and quantitated by dot blot analysis as described under Experimental Procedures.

that of hProPTH-(−6→+84), which was approximately 20 mg/L.

When protein synthesis was inhibited by addition of chloramphenicol 5 h after induction with IPTG (see Figures 3 and 4), immunoreactive hPTH-(1–84) was cleared rapidly: $t_{1/2}$ = 7 min (intact assay) and $t_{1/2}$ = 11 min (C assay). While immunoreactive hPTH-(3–84) was cleared less rapidly, $t_{1/2}$ = 13 min (intact assay) and $t_{1/2}$ = 17 min (C assay), immunoreactive hProPTH-(−6→+84) was much more stable, being cleared with $t_{1/2}$ = 30 min as assessed by both intact and C assays.

Quantitation of hPTH mRNA. The amounts of PTH mRNA in the induced *E. coli* cells harboring pPTH-(3–84), pPTH-(1–84), or pPTH-(−6→+84) did not differ by more than 2-fold one to the other (Figure 5).

Secondary Structure of mRNA Encoded by the Expression Plasmids. The potential secondary structure in the 5′ regions of mRNA encoding either hPTH-(1–84), hProPTH-(−6→+84), or hPTH-(3–84) was predicted by computer modeling. While the initiator AUG codons were exposed in an open loop

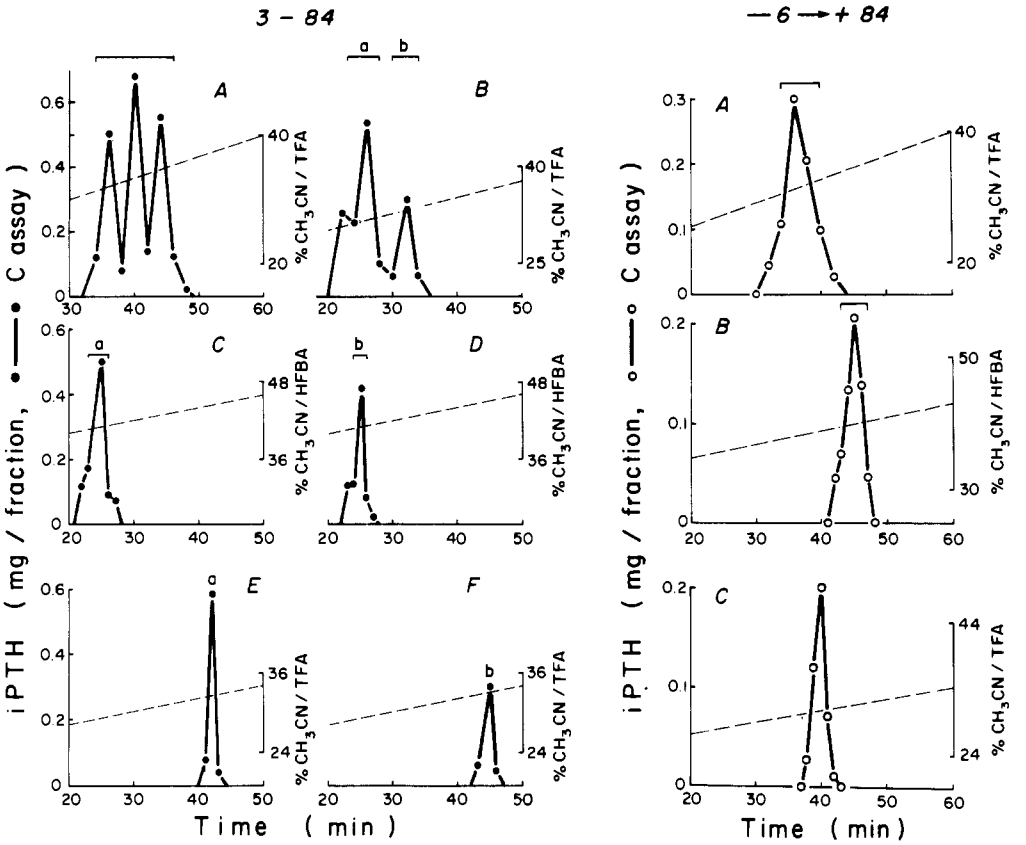

FIGURE 6: Elution profiles of HPLC purification of recombinant hPTH-(3–84) peptide and hPTH-(−6→+84) peptide. *E. coli* cells harboring either plasmid pPTH-(3–84) or plasmid pPTH-(−6→+84) were grown in bulk and acid extracted as described under Experimental Procedures. The extracts were passed over C_{18} Sep-Paks and the eluates processed by HPLC as shown. Left: (Panels A and B) Elution profiles of immunoreactive PTH (iPTH) on the first two reversed-phase HPLC steps eluted with a gradient of acetonitrile/0.1% CF_3COOH (CH_3CN/TFA). The bars indicate the fractions taken at each step and processed further. (Panels C and D) Elution profiles of iPTH on subsequent reversed-phase HPLC eluted with a gradient of acetonitrile/0.13% C_3F_7COOH (CH_3CN/HFBA). The bars indicate the fractions taken and processed further. (Panels E and F) Final purification step showing elution profile of iPTH on reversed-phase HPLC eluted with a gradient of acetonitrile/0.1% CF_3COOH (CH_3CN/TFA). Right: (Panel A) Elution profile of immunoreactive PTH (iPTH) on the initial reversed-phase HPLC step eluted with a gradient of acetonitrile/0.1% CF_3COOH (CH_3CN/TFA). The bar indicates the fractions taken and processed further. (Panel B) Elution profile of iPTH on subsequent reversed-phase HPLC eluted with a gradient of acetonitrile/0.13% C_3F_7COOH (CH_3CN/HFBA). The bar indicates the fractions taken and processed further. (Panel C) Final purification step showing elution profile of iPTH on reversed-phase HPLC eluted with a gradient of acetonitrile/0.1% CF_3COOH (CH_3CN/TFA).

in all three mRNAs, the ribosomal binding site (AGGA) of the 1–84 mRNA was completely buried in a stem structure, whereas that of the −6→+84 mRNA was completely exposed as a single strand, potentially leading to more efficient translation of the ProPTH mRNA. The RBS of the 3–84 mRNA was partially involved in a stem structure intermediate to that of the 1–84 and −6→+84 mRNAs.

Isolation and Purification of PTH-Related Moieties. For the pPTH-(3–84) peptides, approximately 33% of the immunoreactive PTH present in the C_{18} Sep-Pak eluate was recovered at the final purification stage in two major forms designated a and b (see Table I). For the pPTH-(−6→+84) peptide, approximately 63% of the immunoreactive PTH present in the C_{18} Sep-Pak eluate was recovered in the final purification stage as one major form.

HPLC elution profiles of acid extracts of *E. coli* harboring plasmid pPTH-(3–84) are shown in Figure 6. Several incompletely resolving peaks of immunoreactivity were observed on chromatography over a Vydac C_{18} column with a gradient of acetonitrile in 0.1% in CF_3COOH (panel A). Upon rechromatography over a C_{18} μBondapak column with a shallower gradient, two predominant peaks, a and b, of immunoreactivity were observed (panel B). Peaks a and b were pooled separately and purified to homogeneity by sequential chromatography on a reversed-phase column which was eluted with a gradient of acetonitrile in 0.13% C_3F_7COOH (panels C and D, respectively) and on a reversed-phase column which was eluted with a gradient of acetonitrile in 0.1% CF_3COOH (panels E and F, respectively).

Table I: Percentage of iPTH (C Assay) Recovered

purification step	3–84	−6→+84
C_{18} Sep-Pak eluate	100	100
RF-TFA[a]	75	92
RF-TFA	40 (a), 25 (b)	NR[c]
RF-HFBA[b]	25 (a), 17 (b)	70
RF-TFA	21 (a), 12 (b)	63

[a] RF-TFA, reversed-phase chromatography with 0.1% CF_3COOH. [b] RF-HFBA, reversed-phase chromatography with 0.13% C_3H_7COOH. [c] NR, not required for −6→+84 purification.

HPLC elution profiles of acid extracts of *E. coli* harboring plasmid pPTH-(−6→+84) are shown in Figure 6. A single predominant peak of immunoreactivity was eluted from a reversed-phase column with a gradient of acetonitrile in 0.1% CF_3COOH (panel A). This material was purified to homogeneity by sequential chromatography on a reversed-phase column which was eluted with a gradient of acetonitrile in 0.13% C_3F_7COOH (panel B) and on a reversed-phase column eluted with a gradient of acetonitrile in 0.1% CF_3COOH (panel C).

The two major forms, a and b, of the recombinant hPTH-(3–34) peptides showed homogeneous UV profiles when

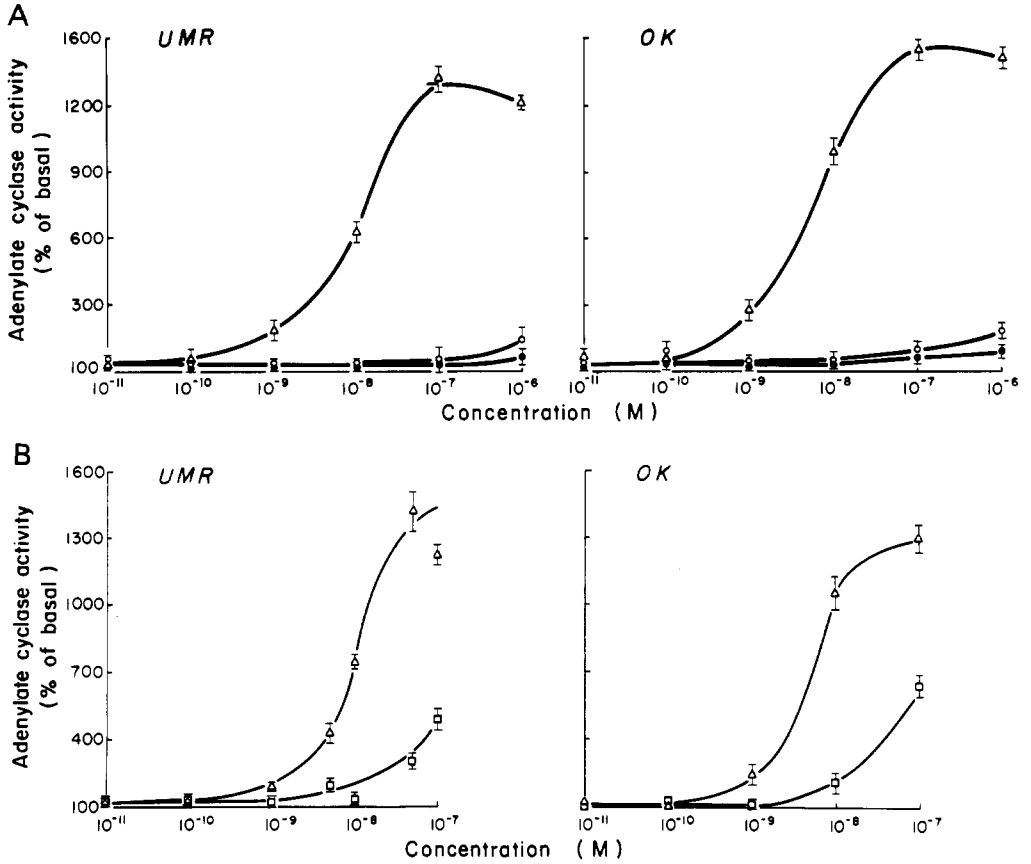

FIGURE 7: Activity of recombinant hPTH-(3–84), fMet-hPTH-(3–84), and fMet-ProPTH-(−6→+84) in skeletal and renal bioassays in vitro. The effects of increasing doses of recombinant hPTH-(3–84) (○), fMet-hPTH-(3–84) (●), fMet-hProPTH-(−6→+84) (□), and standard hPTH-(1–84) (△) on adenylate cyclase activity of UMR 106 osteosarcoma cells and OK opossum kidney cells were assessed as described under Experimental Procedures. Each point is the mean ± SEM of triplicate determinations. Both recombinant 3–84 peptides displayed virtually no agonist activity at all concentrations (upper panels), whereas the −6→+84 peptide had approximately 10% the activity of standard 1–84 (lower panels).

examined by gel filtration HPLC, and each had a molecular weight equivalent to that of standard human PTH-(1–84) (data not shown). The recombinant hPTH-(−6→+84) peptide also was shown to have a molecular weight similar to that of human PTH-(1–84) when analyzed in the same manner (data not shown).

Chemical and Biological Characteristics of PTH-Related Moieties. When each of the purified recombinant hPTH-(3–84) peptides, a and b, was subjected to amino acid analysis, the composition of peptide a was in keeping with that of hPTH-(3–84). The composition of b was similar except for the presence of one extra methionine residue per mole (Table II). The composition of the purified recombinant hPTH-(−6→+84) peptide was similar to that of hProPTH-(−6→+84) except for the presence of one extra methionine residue per mole (Table II).

Each peptide was then subjected to amino acid sequence analysis. Recombinant hPTH-(3–84) peptide a had a sequence beginning at cycle 1 that was identical with that of native human PTH-(3–84) (data not shown). Overall recovery of peptide was greater than 90%. Therefore, the sequence analysis, taken in conjunction with its amino acid composition, assigned peptide a as hPTH-(3–84). Recombinant hPTH-(3–84) peptide b had a sequence, beginning at cycle 2, that was identical with that of native human PTH-(3–84) (data not shown). The residue at cycle 1 was not identified, and the overall recovery of peptide was less than 50%, indicating that the recombinant peptide had a partially blocked NH_2 terminus. Taken in conjunction with the finding of an extra methionine residue on amino acid analysis, this assigned peptide b as formylmethionyl-hPTH-(3–84). Recombinant hPTH-(−6→+84) peptide had a sequence, beginning at cycle 2, that was identical with that of native human ProPTH. The residue at cycle 1 was not identified, and the overall recovery of peptide was less than 50%, indicating that the recombinant peptide had a partially blocked NH_2 terminus. Taken in conjunction with the finding of an extra methionine residue on amino acid analysis, this assigned the recombinant peptide as formyl-methionyl-hProPTH-(−6→+84).

The bioactivities of the purified recombinant peptides were assessed in adenylate cyclase assays performed in osteoblast-derived osteosarcoma cells and in a renal cell line in vitro. Figure 7A shows that both recombinant hPTH-(3–84) and fMet-hPTH-(3–84) displayed virtually no agonist activity at all concentrations tested in both UMR 106 osteosarcoma cell and OK opossum kidney cell adenylate cyclase assays. In the OK opossum kidney cell assay recombinant hPTH-(3–84) was an antagonist, but was approximately 10% as potent as bovine [$Nle^{8,18}Tyr^{34}$]PTH-(3–34)-NH_2, and recombinant fMet-hPTH-(3–84) was even less potent (Figure 8A). In the UMR 106 osteosarcoma assay the hPTH-(3–84) and fMet-hPTH-(3–84) were even less effective as antagonists relative to the bPTH-(3–34) analogue (Figure 8B). The antagonistic properties of synthetic [Tyr^{34}]bPTH-(7–34)-NH_2 and synthetic hPTH-(7–84) were also tested in the OK opossum kidney cell assay (data not shown). Synthetic [Tyr^{34}]bPTH-(7–34)-NH_2 was, like recombinant hPTH-(3–84), approximately 10% as potent as [$Nle^{8,18}Tyr^{34}$]bPTH-(3–34)-NH_2 as an antagonist, and synthetic hPTH-(7–84) was less than 1% as potent.

In both skeletal (UMR) and renal (OK) bioassays the re-

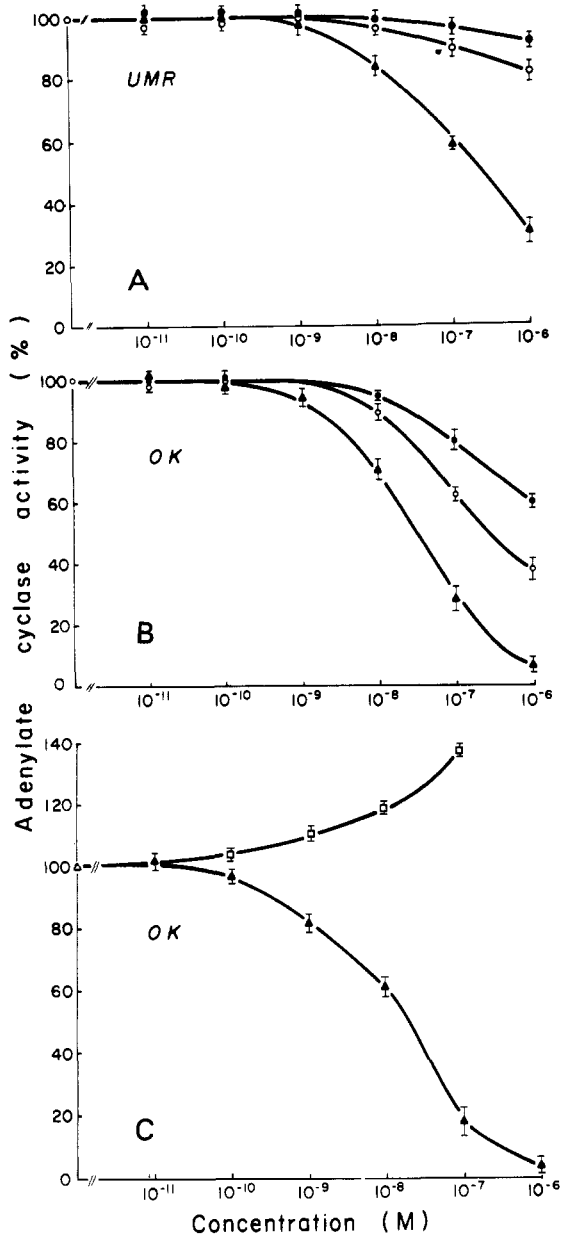

FIGURE 8: Antagonist activity of recombinant hPTH-(3–84) peptides and fMet-hProPTH-(−6→+84) on adenylate cyclase activity bioassays in vitro. The effects of increasing concentrations of recombinant hPTH-(3–84) (○), recombinant fMet-hPTH-(3–84) (●), fMet-hProPTH-(−6→+84) (□), and [Nle^{8,18}Tyr^{34}]bPTH-(3–34)-NH$_2$ (▲) on adenylate cyclase activity stimulated by 5×10^{-9} M hPTH-(1–84) in OK opossum kidney cells and UMR 106 osteosarcoma cells in vitro were assessed as described under Experimental Procedures.

combinant fMet-hProPTH-(−6→+84) had less than 10% of the agonist activity of the hPTH-(1–84) standard (Figure 7B). In both UMR 106 osteosarcoma cells (data not shown) and opossum kidney OK cells (Figure 8), the recombinant fMet-hProPTH-(−6→+84) was without antagonist activity.

Infusion of bPTH-(1–84) into thyroparathyroidectomized rats produced characteristic increases in cAMP excretion and phosphaturia (Figure 9, panels A and B). In addition, the intact molecule maintained or slightly increased plasma calcium and reduced calcium excretion relative to control levels (Figure 9, panels C and D). Infusion of recombinant fMet-hProPTH-(−6→+84) also stimulated increases in cAMP excretion and phosphaturia, and maintained plasma calcium and reduced calcium excretion relative to control (Figure 9). However, the onset of the response was delayed, and the magnitude of the response to fMet-hProPTH-(−6→+84) was reduced, relative to that of bPTH-(1–84). The responses to infusion of either synthetic [Nle^{8,18}Tyr^{34}]bPTH-(3–34)-NH$_2$ or recombinant hPTH-(3–84) were generally not significantly different from control values although there was some evidence of partial agonist activity of these peptides in terms of urinary cyclic AMP excretion and maintenance of plasma calcium levels (Figure 9, panels A and C).

When a 10-fold molar excess of amino-terminally truncated peptides was coinfused with intact PTH into thyroparathyroidectomized rats, there was no evidence of antagonist behavior by these peptides. In general, the responses to infusion of either [Nle^{8,18}Tyr^{34}]bPTH-(3–34)-NH$_2$ or recombinant hPTH-(3–84) with bPTH-(1–84) were no different from those seen with bPTH-(1–84) alone (Figure 10). However, increases in nephrogenous cAMP excretion and phosphaturia were seen at some time points during the coinfusion of bPTH-(1–84) and the 3–34 or 3–84 peptides relative to bPTH-(1–84) alone (Figure 10, panels A and B). This may indicate more effective delivery of the hPTH-(1–84) by coinfusion with the additional peptides due to reduced "stickage" or enhancement of the efficacy of hPTH-(1–84) during coinfusion due to reduced degradation.

Discussion

Both of the expression plasmids used in this study were prepared from plasmid pPTH-84 (Rabbani et al., 1988b). In the case of the plasmid directing the synthesis of hPTH-(3–84), a "duplex cross-over linker" technique (Sung et al., 1986a,b) was used to link the hPTH-(3–84) directly to the lac operator. In the case of the expression plasmid encoding hProPTH-(−6→+84) a "single-stranded cross-over linker" technique (Sung et al., 1989; Sung & Zahab, 1987) was employed. The mechanism for recombination directed by a single-stranded cross-over linker, such as PRO-1 used here, may be a variation of pathways originally proposed for related recombination in other cell lines (Lin et al., 1984). During transformation, an exonucleolytic breakdown of the targeted terminus exposed the complementary strand for homologous intramolecular pairing with the linker (Radding, 1973). Further nucleolytic degradation released any unpaired, redundant sequence beyond the region of annealing (Cassuto & Radding, 1971). An in vivo DNA polymerization would subsequently repair the gap at the single-stranded region to yield a circular plasmid with nicks. However, this ability to direct in vivo DNA polymerization appeared to be limited, as it failed to generate mutants if the gap to be filled in was wider than 22 bases, as shown in other studies (Sung et al., 1989; Sung & Zahab, 1987). This limited capability indicated that the initial exonucleolytic reaction may be controlled or directed (Radding, 1973; Cassuto & Radding, 1971) such that the degradation would not be so extensive as to generate a gap which was too wide to be repaired. We were successful, however, in the study described here, in exploiting this repair mechanism in the generation of expression plasmid hProPTH-(−6→+84).

In previous studies using an expression plasmid which directed the synthesis of hPTH-(1–84) under the control of the *E. coli* lac promoter, a relatively modest level of expression was obtained (Rabbani et al., 1988b). However, placement of the synthetic hPTH-(1–84) gene under the control of several other, potentially stronger, *E. coli* promoters brought about no significant increase in the level of iPTH obtained (unpublished observations). This has also been the finding of others (Wingender et al., 1989). In the studies described here we found some increase in the level of expression of hProPTH-(−6→+84). All three plasmids contain identical nucleotide sequences upstream of the initiator codon ATG,

Table II: Amino Acid Composition of HPLC-Purified Recombinant hPTH Peptides

	recombinant hPTH-(3–84)							
	peptide a		peptide b			recombinant peptide −6→+84		
amino acid	residues/mol	nearest integer	residues/mol	nearest integer	hPTH (3–84)[a]	residues/mol	nearest integer	hProPTH[a]
Lys	9.4	9	8.9	9	9	12.2	12	12
His	4.2	4	3.6	4	4	4.4	4	4
Arg	5.2	5	5.4	5	5	6.2	6	6
Asp[b]	9.7	10	10.3	10	10	9.8	10	10
Thr	1.3	1	1.4	1	1	1.1	1	1
Ser	5.8	6	6.2	6	6	8.2	8	8
Glu[b]	11.2	11	10.8	11	11	10.9	11	11
Pro	3.4	3	2.7	3	3	3.4	3	3
Gly	4.2	4	3.9	4	4	3.9	4	4
Ala	6.8	7	7.1	7	7	7.3	7	7
Val	7.3	7	6.8	7	7	9.1	9	9
Met	2.1	2	2.8	3	2	2.7	3	2
Ile	0.9	1	1.2	1	1	0.9	1	1
Leu	9.8	10	10.4	10	10	10.2	10	10
Tyr	0.3	0	0.2	0	0	0.4	0	0
Phe	0.8	1	1.4	1	1	0.9	1	1

[a] Predicted amino acid composition. [b] Amide nitrogen and tryptophan not determined.

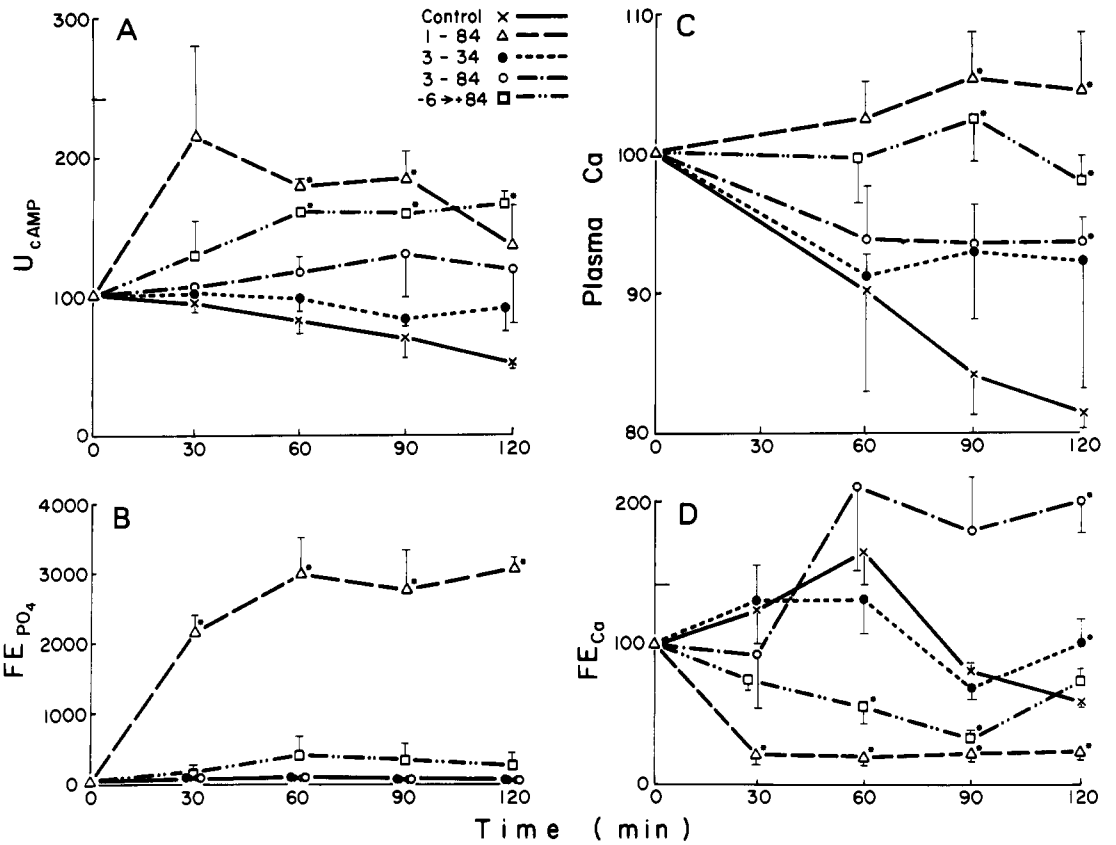

FIGURE 9: Effect of infusion into thyroparathyroidectomized rats of recombinant hPTH peptides: assessment of agonist activities. bPTH-(1–84) (1 nmol/h) (△), recombinant hPTH-(3–84) (1 nmol/h) (○), synthetic bPTH-(3–34) (1 nmol/h) (●), recombinant fMet-hProPTH-(−6→+84) (1 nmol/h) (□), and vehicle alone (×). Each point is the mean ± SEM of 3–12 determinations. Parameters measured were cAMP excretion in picomoles per milliliter of glomerular filtrate (U_{cAMP}; panel A), the fractional excretion of phosphate (FE_{PO_4}; panel B), plasma calcium (panel C), and the fractional excretion of calcium (FE_{Ca}; panel D). Data are expressed as percentage of the control value for each peptide infused. Asterisks indicate significant differences ($P < 0.05$, by ANOVA), at the corresponding times, from the values obtained during infusion of vehicle alone. Time zero is the time of initiation of all infusions, as described under Experimental Procedures.

which corresponds to the lac promoter and ribosomal binding site. Similar amounts of mRNA are transcribed from each of the different gene constructs as assessed by dot blot analysis. Therefore, differences in the levels of expression as assessed by iPTH measurement may be due to differences in the efficiency of translation of the mRNA as suggested by secondary structure analysis and/or the stability of the product. These two factors may be operating in the studies described here.

The greater stability of the hProPTH-(−6→+84) peptide was more evident when degradation rates of hProPTH and of the other recombinant peptides were examined with an intact molecule assay rather than a C assay. The discordance between degradation rates of hPTH-(1–84) and of hPTH-(3–84) determined by intact and C assay was indicative of more rapid turnover of the NH_2 terminus. This in turn could be a function of the ease with which the formylmethionyl residue is removed; i.e., if the fMet group is retained, some protection may be afforded against subsequent proteolytic cleavage. The amino-terminal formylmethionine is removed enzymatically by a methionylaminopeptidase, and the relative

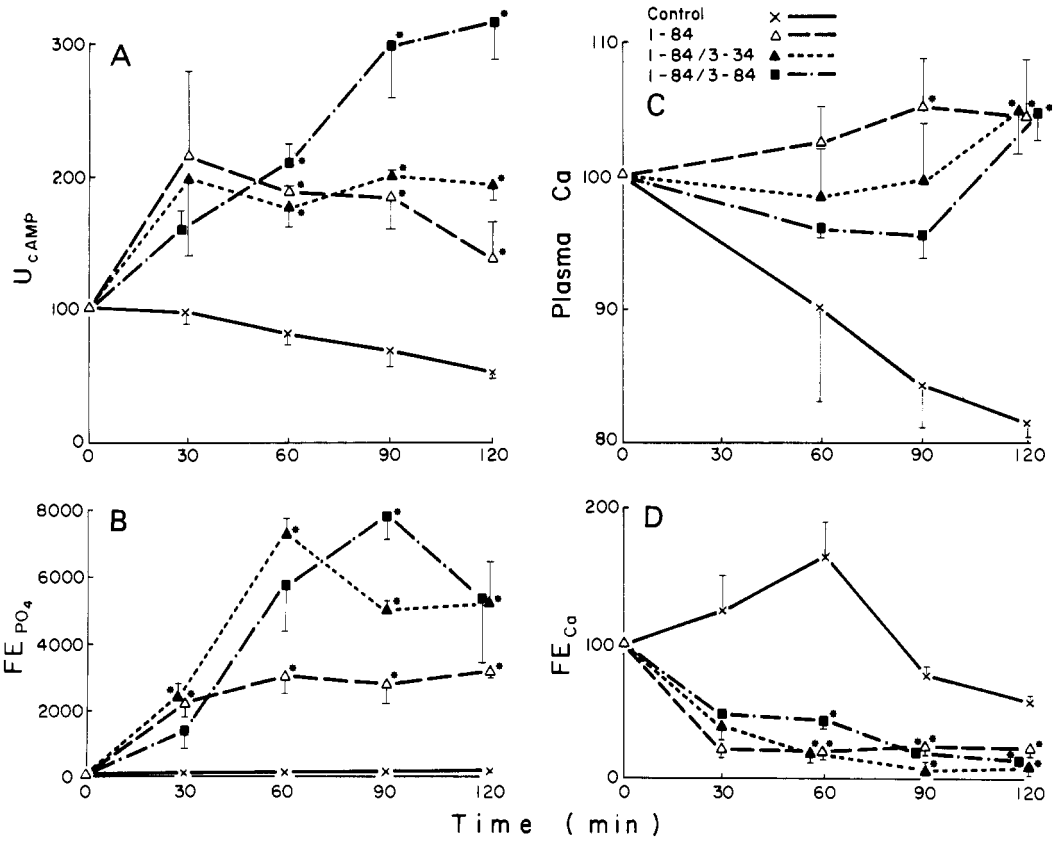

FIGURE 10: Effect of infusion into thyroparathyroidectomized rats of PTH peptides: assessment of antagonist activity of recombinant hPTH-(3–84). bPTH-(1–84) (1 nmol/h) (△), bPTH-(1–84) (1 nmol/h) and synthetic bPTH-(3–34) (10 nmol/h) (▲), bPTH-(1–84) (1 nmol/h) and recombinant hPTH-(3–84) (10 nmol/h) (■), and vehicle alone (×). For details of parameters measured, see legend to Figure 9 and Experimental Procedures.

efficiency with which this occurs is determined in part by the nature and size of the neighboring amino acid (Ben-Bassat et al., 1987). This was borne out by the fact that, for molecules such as hPTH-(1–84) and hPTH-(3–84) with serine, a small amino acid, at positions +1 and +3, respectively, the formylmethionyl group was removed in over 60% of the recombinant molecules. However, for molecules such as hProPTH-(−6→+84) with a more bulky lysine residue at amino acid −6, the formylmethionyl group was resistant to removal. This may have accounted in part for the greater NH_2-terminal stability of the hProPTH molecule. Changes in conformation or charge properties brought about by NH_2-terminal extension in the hProPTH molecule are other potentially important factors contributing to stability which cannot at this time be excluded. Recently, the expression of hPTH-(1–84) in a bacterial system which excretes the peptide into the culture medium has been described (Høgset et al., 1990b). This holds promise as a method to overcome intracellular degradation and incomplete removal of the amino-terminal formylmethionyl residue.

The extended analogue, fMet-hProPTH-(−6→+84), was shown to have less than 10% of the bioactivity of hPTH-(1–84) in both skeletal and renal in vitro adenylate cyclase assays. This is consistent with previous studies with synthetic NH_2-terminal PTH analogues which demonstrated that bProPTH-(−6→+34) has approximately 5% of the activity of bPTH-(1–34) (Peytremann et al., 1975). Furthermore, it has recently been reported that an NH_2-terminally extended recombinant hPTH analogue, fMet-Gly-hPTH-(1–84), was without adenylate cyclase stimulating activity in an in vitro rat osteosarcoma cell membrane assay (Høgset et al., 1990a). In our studies, in vivo, the potency of fMet-hProPTH was more pronounced than that in vitro. This discrepancy between in vivo and in vitro biopotency has also previously been noted for bProPTH-(−6→+34) and may reflect the greater capacity of endopeptidases to cleave the prohormone–hormone bond and generate an active hormonal product in vivo than in bioassay systems in vitro. Finally, the recombinant fMet-hProPTH-(−6→+84) had no in vitro antagonist activity in either renal or skeletal adenylate cyclase assays. Consequently, although fMet-hProPTH-(−6→+84) contains the entire receptor binding sequence, the overall results suggest that conformational changes brought about by extension of the molecule reduce effective interactions with the PTH receptor.

Both deleted recombinant analogues, hPTH-(3–84) and fMet-hPTH-(3–84), had virtually no agonist activity in renal and skeletal adenylate cyclase assays in vitro, thus underscoring the critical importance of the NH_2-terminal region in triggering cyclic AMP production. The in vivo role of the NH_2 terminus in mediating calcium and phosphate transport as well as cyclic AMP production was also emphasized by the absence of the in vivo activity of hPTH-(3–84). Therefore, the full-length hormone demonstrates a similar requirement for integrity of the NH_2-terminal sequence, as does the analogue based on the 1–34 sequence. The in vitro antagonist studies which demonstrated that hPTH-(3–84) was less potent than the PTH-(3–34) analogue were, in contrast, more difficult to anticipate. Thus one previous study has reported that hPTH-(3–84) was more potent as an antagonist (in an in vitro renal cytochemical bioassay) than the PTH-(3–34) analogue (Born et al., 1988). Neverthless, the bulk of existing evidence suggests that extension of the NH_2-terminal fully active fragment toward the carboxyl end reduces both agonist and antagonist activity. Thus PTH-(1–84) has been reported to be somewhat less potent than PTH-(1–34) in assays in vitro (Demay et al., 1985; Goldman et al., 1988), and extension of NH_2-terminally truncated analogues from amino acid 34 to amino acid 38 has been reported to result in reduced antagonist

activity and diminished receptor binding in vitro (Goldman et al., 1988). Our studies with the 3–84 and the 3–34 analogue, as well as with the 7–84-amide and 7–34-amide, are therefore consistent with these latter reports and point to a role for the 34–84 region of PTH in modulating the capacity of the NH_2-terminal region to interact with its receptor.

In assessing the biological activities of recombinant peptides, the potential role of posttranslational modifications should be borne in mind. In the case of PTH little is known concerning the posttranslational modification of its amino acid sequence. It is not thought to be glycosylated, and although phosphorylation of both ProPTH and PTH has been shown to occur in vitro (Rabbani et al., 1984), the influence of this process on bioactivity remains undefined. Bacterial expression systems such as the one used here are not thought to carry out posttranslational modifications of recombinant peptides to any significant extent. The fact that the recombinant hPTH-(1–84) has the full biological and immunological activity expected of the native hormone (Rabbani et al., 1988b) argues against any posttranslational modification of the recombinant hPTH by the bacterial expression system being important in affecting biological activity.

Although the present studies have focused on biological functions resulting from interaction of PTH with an NH_2-terminal responsive receptor linked to adenylate cyclase activation and calcium and phosphate transport, recent studies have demonstrated the presence of binding sites for the midregion and carboxyl ends of the molecule (McKee & Murray, 1985; Demay et al., 1985) and have reported effects of PTH on cellular proliferation (Schluter et al., 1989) and differentiation (Murray et al., 1989). The deletion of regions of the 1–84 molecule related to calcium and phosphate transport might not disrupt such functions and might be preferable in designing analogues where anabolic effects of PTH predominate. The availability of analogues such as hPTH-(3–84) may therefore be useful to test such strategies.

Acknowledgments

We thank Isabel Bolivar and Miren Gratton for assistance with aspects of these studies, Dr. Hugh Bennett for advice and amino acid analyses, and Wendy Campbell, Shelley Hall, and Diane Allen for preparation of the manuscript.

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