THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 266, No. 5, Issue of February 15, pp. 2831-2835, 1991
Printed in U.S.A.

Specific Degenerate Codons Enhanced Selective Expression of Human Parathyroid Hormone in *Escherichia coli**

(Received for publication, June 25, 1990)

Wing L. Sung‡, Diana M. Zahab, Jean R. Barbier, David Watson, Makoto Yaguchi, Witold Neugebauer, and Gordon E. Willick

From the Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A OR6, Canada

Specific degenerate codons in the amino-terminal region of a synthetic human parathyroid hormone (PTH) gene exerted dramatic effects on both products and yield of expression of this 84-amino acid polypeptide in *Escherichia coli*. With adenine-rich degenerate codons constituting the PTH-(1–5) region, intact PTH has been expressed as the only PTH product at 6.5 mg/liter. In contrast, with guanine-rich degenerate codons, the predominent product was analogue PTH-(8–84). Use of cytosine- or thymine-rich degenerate codons generated only a small amount of immunoreactive product (0.2 mg/l).

With the amino terminal region reconstituted with adenine-rich degenerate codons, the mid and carboxyl regions of the synthetic gene were also reconstructed to imitate the *E. coli*-favored codon degeneracy. Expression yielded the intact PTH at 20 mg/liter. Gel electrophoresis and Western blots, with antibodies specific to the amino or carboxyl terminus of PTH, indicated only a single PTH-related polypeptide, with the same mobility as a synthetic intact PTH sample. Amino acid sequencing, composition analysis, mass spectrometry, and the adenylate cyclase bioassays confirmed the purified product as the processed intact PTH.

Human parathyroid hormone, a polypeptide of 84-amino acid residues (Hendy *et al.*, 1981), is a major regulator of serum calcium. Synthetic fragments have demonstrated characteristic effects in bone metabolism (Reeve *et al.*, 1980) that may potentially be useful in the treatment of some bone disorders. Various attempts to produce the intact polypeptide via expression of the prepro-PTH[1] cDNA have been reported. However, the prepro-PTH sequence was unable to facilitate secretion and processing of PTH in yeast (Born *et al.*; 1987a) and *Escherichia coli* (Born *et al.*, 1987b). Substitution with a bacterial leader sequence yielded a secreted immunoreactive mixture (1 mg/liter) of intact PTH and fragments (Hogset *et al.*, 1990). Direct expression of the PTH cDNA (minus the prepro sequence) intracellularly generated immunoreactiove PTH at 0.2 mg/liter in *E. coli* (Breyel *et al.*, 1984). It was postulated that the low yield was due to instability of both PTH and its mRNA (Morelle *et al.*, 1988).

In our laboratory, a synthetic PTH gene was assembled and directly expressed in *E. coli* to yield immunoreactive PTH at 0.2 mg/liter (Rabbani *et al.*, 1988). Analysis of this mixture revealed fragment PTH-(8–84), fMet-PTH and intact PTH. Recently the synthetic gene has been redesigned with specific degenerate codons, resulting in dramatically improved efficiency of expression (20 mg/liter) and selective production of intact PTH or fragment PTH-(8–84). Intact PTH was isolated, characterized, and assayed for bioactivity.

DISCUSSION[2]

An efficient expression system for human PTH has been developed, through (i) the usage of adenine-rich degenerate codons in the amino-terminal domain, (ii) adaptation of the "*E. coli*-like" codon degeneracy in the mid/carboxyl-terminal domains, and (iii) selection of an appropriate expression host. The choice of degenerate codons or the specific nucleotide composition of the amino-terminal coding sequence exerts a strong influence in both the efficiency of expression and the expressed product (or analogue).

Other studies have previously demonstrated that expression of some genes can be improved by elimination (or weakening) of secondary structure of mRNA (Hall *et al.*, 1982). In the PTH mRNA of our plasmids pPTH-CC and pPTH-TT (Table 1), indeed hairpin structure can potentially be formed between the A,G-rich ribosome-binding site and the amino-terminal region with respective ΔG values of -7.0 and -9.6 kcal (Tinoco *et al.*, 1973), to interrupt the translation process. The A-rich degenerate codons in the PTH-(1–5) region of plasmid pPTH-AA (Table 1), might have weakened such secondary structure ($\Delta G = -3.2$ kcal) and consequently improved PTH expression. However, such mechanism is inadequate to explain the poor expression by plasmid pPTH-84c (Table 1), which has an identically weakened secondary structure in the PTH mRNA ($\Delta G = -3.4$ kcal). The efficient PTH production by the plasmids pPTH-AA and pPTH-AA-Eco (Tables 1 and 2) also contradicts earlier conclusions that tandem repeats of rare degenerate codons (Varenne and Lazdunski, 1986) and their proximity to the initiation codon would dramatically reduce the maximal level of protein synthesis (Chen and Inouye, 1990). Clustered at the PTH amino-terminal region of both plasmids, the five codons TCA-1, GTA-2, TCA-3, ATA-5, and TTA-7 are rare degenerate codons in *E. coli* (Chen *et al.*, 1982).

* This is National Research Council of Canada Publication 31886. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom reprint requests should be addressed. Tel.: 613-990-0815.

[1] The abbreviations used are: PTH, human parathyroid hormone; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; IPTG, isopropyl-D-thiogalactoside; RIA, radioimmunoassay.

[2] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–7, Tables 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

Comparison of the plasmids pPTH-AA, pPTH-CompB, pPTH-AA-Eco, pPTH-CompE-Eco, pPTH-GG, and pPTH-GG-Eco with the other plasmids much less efficient in PTH expression (Tables 1 and 2), indicates that the efficiency of PTH expression instead might be determined by the nucleotide (adenine, and likely to a small extent, guanine) composition of the amino-terminal coding sequence.

Selection of degenerate codons in PTH gene design also influenced the products of expression. Though designed to produce intact PTH, plasmids pPTH-GG and pPTH-GG-Eco selectively generated the short fragment PTH-(8–84), with the intact PTH only as a minor product. Although this short analogue could conceivably be derived from intact PTH through (unknown) specific proteolysis, the mere difference of four silent point mutations between plasmids pPTH-GG-Eco and pPTH-AA-Eco supported a previously proposed hypothesis of internal initiation of translation (Born *et al.*, 1987b). In plasmid pPTH-GG-Eco, the codons 3, 4, and 5, TCG-GAG-ATA can potentially constitute a strong ribosome-binding site (underlined) to initiate a competing translation from ATG-8 to yield PTH-(8–84). In another plasmid pPTH-84c, with a potentially weaker ribosome-binding site (TCT-GAG-ATC) in the same region, a 2:1 mixture of PTH and PTH-(8–84) was produced (Rabbani *et al.*, 1988). This hypothesis is also consistent with the exclusive production of intact PTH by both plasmids pPTH-AA and pPTH-AA-Eco, which possess no similar internal ribosome-binding site. However, the precise mechanism remains to be confirmed.

As for the production of the intact PTH, the residue *Ser*I adjacent to fMet, has a small radius of gyration essential for the efficient removal of fMet residue from the nascent polypeptide (Sherman *et al.*, 1985).

The present approach of direct expression of a synthetic gene has successfully yielded biologically active, intact PTH. The extraction and purification procedures would efficiently generate adequate amounts for future studies.

Acknowledgments—We thank Stephanie Allard, Marc Lafontaine, and Perry Fleming for assistance in the expression and fermentation studies (National Research Council of Canada), Dr. Y. Konishi of Biotechnology Research Institute, for obtaining mass spectra, and Jennifer Pendlebury of Allelix Biopharmaceutics (Mississauga, Canada) for conducting the bioassay studies.

REFERENCES

Born, B., Freeman, M., Bornstein, W., Rapoport, A., Klein, R. D., Hendy, G. N., Khorana, H. G., Rich, A., Potts Jr., J. T., and Kronenberg, H. M. (1987a) *J. Bone Miner. Res.* **2,** 353–360

Born, B., Freeman, M., Hendy, G. N., Rapoport, A., Rich, A., Potts Jr., J. P., and Kronenberg, H. M. (1987b) *Mol. Endocrinol.* **1,** 5–14

Breyel, E., Morelle, G., Auf'mkolk, B., Frank, R., Blocker, H., and Mayer, H. (1984) *3rd Congr. Eur. Biotech.* **111,** 363–369

Chen, G.-F. T., and Inouye, M. (1990) *Nucleic Acids Res.* **18,** 1465–1473

Chen, H. R., Dayhoff, M. O., Barker, W. C., Hunt, L. T., Yeh, L.-S., George, D. G., and Orcutt, B. C. (1982) *DNA* **1,** 365–374

Hall, M. N., Gabay, J., Debarbouille, M., and Schwartz, M. (1982) *Nature* **295,** 616–618

Hendy, G. N., Kronenberg, H. M., Potts Jr., J. T., and Rich, A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7365–7369

Hirs, C. H. W. (1967) *Methods Enzymol.* **11,** 197–199

Hogset, A., Blingsmo, O. R., Saether, O., Gautvik, V. T., Holmgren, E., Hartmanis, M., Josephson, S., Gabrielsen, O. S., Gordeladze, J. O., Alestrom, P., and Gautvik, K. M. (1990) *J. Biol. Chem.* **265,** 7338–7344

Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038

Morelle, G., and Mayer, H. (1988) *Biochim. Biophy. Acta* **950,** 459–462

Posnett, D. N., McGrath, H., and Tam, J. P. (1988) *J. Biol. Chem.* **263,** 1719–1725

Rabbani, S. A., Yasuda, T., Bennett, H. P. J., Sung, W. L., Zahab, D. M., Tam, C. S., Goltzman, D., and Hendy, G. N. (1988) *J. Biol. Chem.* **263,** 1307–1313

Reeve, J., Meunier, P. J., Parsons, J. A., Bernaut, M., Bjvoet, O. L. M., Courpron, P., Edouard, C., Klenerman, L., Neer, R. M., Reneir, J. C., Slovick, D., Vismans, F. J. F. E., and Potts Jr., J. T. (1980) *Br. Med. J.* **280,** 1340–1344

Sherman, F., Stewart, J. W., and Tsunasawa, S. (1985) *BioEssays* **3,** 27–31

Shine, J., and Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 1342–1346

Simpson, R. J., Neuberger, M. R., and Liu, T.-Y. (1976) *J. Biol. Chem.* **251,** 1936–1940

Stewart, J. M. and Young, J. D. (1984) *Solid Phase Peptide Synthesis,* 2nd Ed., pp. 1–40, Pierce Chemical Co., Rockford, IL

Sung, W. L., Zahab, D. M., Yao, F.-L., and Tam, C. S. (1986a) *Biochem. Cell Biol.* **64,** 133–138

Sung, W. L., Zahab, D. M., MacDonald, C. A., and Tam, C. S. (1986b) *Gene (Amst.)* **47,** 261–267

Tinoco Jr., I. T., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M., and Gralla, J. (1973) *Nature New Biol.* **246,** 40–41

Towbin, H., Stachelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354

Varenne, S., and Lazdunski, C. (1986) *J. Theor. Biol.* **120,** 99–110

Wingender, E., Bercz, G., Blöcker, H., Frank, R., and Mayer, H. (1989) *J. Biol. Chem.* **264,** 4367–4373

Supplementary Material To
Specific Degenerate Codons Enhanced Selective Expression of
Human Parathyroid Hormone in *Escherichia coli*.

By Wing L. Sung, Diana D. Zahab, Jean R. Barbier, David Watson,
Makoto Yaguchi, Witold Neugebauer, and Gordon E. Willick.

EXPERIMENTAL PROCEDURES

Materials. *E. coli* strains JM103 (Δ(*lac pro*), *thi*, *str A*, *sup E*, *end* A_1, *sbc B*, *hsd R*, *F'tra* D36, *pro* AB, *lac* I^Q, Z M15), HB101 (F^-, *hsd S20* (r_B^-, m_B^-), *rec A13*, *ara-14*, *pro A2*, *lac Y1*, *gal K2*, *rps L20* (Sm^r), *xyl-5*, *mtl-1*, *sup E44*, λ^-), Y1091 (*Δlac* U169; *pro* A^+, *Δ lon*, *ara* D139, *str A*, *sup* F [*trp* C22::Tn 10]) (Clontech Lab, Palo Alto, CA) were used as the transformation and expression hosts. Synthetic oligonucleotides were prepared with an Applied Biosystem DNA Synthesizer, Model 380B. Plasmid pUC8 and enzymes were purchased from Bethesda Research Laboratories. Plasmid pPTH-84 has previously been prepared via insertion of a synthetic PTH gene between the *Eco*RI and *Hind* III sites of plasmid pUC8 (Sung *et al.*,1986a). The two-site Allegro Intact PTH RIA kit was from Nichols Institute Diagnostics (San Juan Capistrano, CA). Synthetic human PTH-(1-84) was from Bachem Corp., Torrance, CA.

Construction of Expression Plasmids. Plasmids pPTH-AA, pPTH-GG, pPTH-CC, pPTH-TT, pPTH-compB, pPTH-WA, pPTH-wxA and pPTH-hA (Table 1) were all prepared in the same manner. Construction of plasmid pPTH-AA is described as an example (Figure 1). Oligonucleotides COL-1, P1AA, P2B, P3, P6, P7B, and P8AA were phosphorylated and ligated to the *Eco*RI/*Pst*I- linearized plasmid pPTH-84 via a well-established protocol (Rabbani *et al.*, 1988). After transformation of *E. coli* JM103, 14% of the transformants were identified to possess plasmid pPTH-AA by DNA hybridization and nucleotide sequencing.

The protocol for the construction of plasmid pPTH-AA-Eco was identical to the published procedure (Sung *et al.*, 1986a). It involved ligation of the phosphorylated oligonucleotides P103b, P104, P105, P106, P201, P202, P203 and P204a with the *Pst*I/*Hind*III-linearized plasmid pPTH-AA (Figure 2).

Plasmid pPTH-(29-84)-Eco was prepared via ligation of the PTH-(29-84) insert, which was previously isolated from *Pst*I/*Hind*III-linearized plasmid pPTH-AA-Eco, with plasmid pUC8 cut at the same sites. Substitution of the same insert in plasmids pPTH-GG, pPTH-wA and pPTH-hA yielded plasmids pPTH-GG-Eco, pPTH-wA-Eco, and pPTH-hA-Eco (Table 2).

Plasmids pPTH-A-Eco~(8-84), pPTH-A-Eco~(18-84), and pPTH-CompE-Eco (Table 2) were prepared via ligation and recombination of appropriate oligonucleotides with the *Eco*RI/*Pst*I-cut plasmid pPTH-(29-84)-Eco, as in the construction of plasmid pPTH-AA.

Expression in E. coli. One liter of 2YT + 1% CA medium (16g Bacto-tryptone, 10g Bacto-yeast extract, 10g NaCl, and 10g Casamino acid in 1 liter) containing ampicillin (100 mg/l) was innoculated with a 10 ml overnight growth of *E. coli* Y1091 or HB101 transformants and incubated at 37°C with shaking. Cells were harvested by centrifugation after 9 h. For *E. coli* JM103 transformants, IPTG (final concentration 0.7 mM) was added after 1.5 h of initial growth. For obtaining a whole cell lysate, cells from 5 ml culture were sonicated in 1.25 ml of 1% SDS.

Extraction Procedure. Cells were sonicated (1 min, pulsed) at 4°C in a mixture (1 ml/g) of 1 M HCl containing 1% (w/v) NaCl, and 1% (v/v) TFA (Rabbani *et al.*, 1988) and centrifuged. The cell debris was reextracted. The extracts were pooled.

HPLC. The extract was adjusted to pH 3.8 with NaOH, diluted with water (4:1), and applied to a HL 10/10 Mono S column (Pharmacia). The column was eluted with a gradient of 0-2 M NaCl in 50 mM formic acid (pH 3.8) and fractions were evaluated by RIA. Immunoreactive fractions were pooled and applied to a 1 x 25 cm C_{18} silica (10 μm) column (Vydac) (Rabbani *et al.*,1988). It was then eluted with a 1%/min gradient of 0.1% TFA/acetonitrile in 0.1% TFA/water. Immunoreactive fractions containing mainly intact PTH were combined and lyopholized.

Antibodies Specific to Amino and Carboxyl Termini. PTH-(69-84) amide was synthesized on methybenzhydrylamine resin, using tBoc chemistry (Stewart and Young, 1984). PTH-(1-17) was constructed on a branched lysine core as described (Posnett *et al.*, 1988). The core was constructed on a phenylacetamidomethyl resin, with a β-alanyl spacer, and using bis-tBoc-Lys. Rabbit antibodies were developed directly to the PTH-(1-17)-Lys complex and to PTH-(69-84) coupled to keyhole Limpet hemocyanin. The antibodies were affinity purified by passage through a column of PTH-(1-17) coupled to AffiGel 15 or PTH-(69-84) coupled to AffiGel 10.

Immunoblots. Western blots (Towbin *et al.*,1979) were saturated with 10% fetal calf serum, then reacted with either rabbit anti-PTH-(1-17) or anti-PTH-(69-84) antibodies. Final development was with anti-rabbit IgG conjugated to alkaline phosphatase.

Amino Acid Sequencing. PTH samples (500 pmole) on polyvinylidene difluoride membrane were analysed via gas-phase sequencing as described (Matsudaira, 1987). PTH sample was also digested with endoproteinase Asp-N (Boehringer Mannheim). The resulting peptides, separated by HPLC on C_{18} silica using a 1%/min gradient of acetonitrile in 0.1 % TFA/water, were sequenced as above.

Amino Acid Composition Analysis. Amino acid composition analyses of protein and of purified peptides were performed with a Durrum D-500 Analyzer. Samples (100 μg) were hydrolyzed *in vacuo* at 110°C in 6 N HCl for 24, 48 and 72 h and the data extrapolated to 0 h to correct for hydrolytic losses. Tryptophan was determined following hydrolysis in 4 N methane sulphonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 20 h *in vacuo* (Simpson *et al.*, 1976). The combined cystine and cysteine content was determined after oxidation to cysteic acid (Hirs, 1976) and hydrolysis in 6 N HCl at 110°C for 24 h.

Mass Spectrometry. IonSpray mass spectra of the purified recombinant PTH-(1-84) was obtained by the API III LC/MS System with an ionspray interface (SCIEX, Mississauga, ONT).

Bioassays. Adenylate cyclase assays of recombinant PTH-(1-84) were performed *in vitro* in cloned rat osteosarcoma cells (UMR 106) as described previously (Rabbani *et al.*,1988). The standards used were synthetic hPTH-(1-84) and hPTH-(1-34).

RESULTS

Effect of different amino-terminal nucleotide sequences on the expression of PTH. A series of synthetic PTH genes were designed to possess maximum numbers of adenine(A), cytosine(C), guanine(G), or thymine(T) in the first five codons at the amino-terminus, as permitted by codon degeneracy, without mutating the polypeptide sequence. Precurser plasmid pPTH-84, possessing a synthetic PTH gene constituted with yeast-favored codons (Sung *et al.*, 1986a), was linearized at the *Eco*RI and *Pst*I sites for the deletion of its PTH-(1-28) region. Oligonucleotides constituting the new PTH-(1-28) nucleotide sequence were ligated with the *Pst*I end of the plasmid (Figure 1). Through an intramolecular recombination, the crossover linker of the oligonucleotides recombined with the *lac* ribosome-binding site upstream for the circularization of the plasmid (Sung *et al.*, 1986b). The reconstructed PTH gene was under the direct control of the *lac* promotor. In this way, plasmids pPTH-AA, pPTH-CC, pPTH-GG, and pPTH-TT, enriched with a specific nucleotide (A, C, G, and T, respectively) in their PTH-(1-5) regions, were constructed. The efficiencies of expression of the *E. coli* JM103 transformants possessing these plasmids, upon induction by IPTG, were compared (Table 1).

Table 1. Expression of PTH genes possessing different amino-terminal nucleotide sequences in *E. coli* strain JM103.

PTH gene -containing plasmids	PTH[a] mg/l	N-terminal coding sequences[b] 1 2 3 4 5 Ser Val Ser Glu Ile
pPTH-AA	3.9	TCA GTA TCA GAA ATA
pPTH-wA[c]	3.4	
pPTH-wxA[d]	3.5	
pPTH-CC	0.15[e]	C C C C
pPTH-GG	10[f]	G G G G
pPTH-TT	0.25[e]	T T T T
pPTH-CompB	1.1	AGT T AGT T
pPTH-hA	0.3[e]	T G AGT
pPTH-84c[g]	0.19[h]	G T T G C

a Estimated by the Allegro RIA.
b Nucleotide sequence encoding the PTH-(1-5) region of pPTH-AA is presented. For other plasmids, only nucleotides different from pPTH-AA are presented in this Table. Codon differences in other regions are stated individually.
c GTA-21 and TTA-24.
d AAA-13, 26, 27, TTA-15, 24, TCA-17 and GTA-21.
e Nature of the immunoreactive PTH not determined.
f Mixture of PTH-(1-84) and PTH-(8-84). See Fig. 4.
g Previously synthesized (Rabbani *et al.*, 1988).
h Mixture of PTH and PTH-(8-84) in ratio of 2:1.

Radioimmunoassay indicated that the transformant JM103:pPTH-AA produced PTH at 3.9 mg/l culture, a 20-fold increase as compared to 0.2 mg/l by the previously constructed vector pPTH-84c (Rabbani *et al.*,1988) (Table 1). Transformants with plasmid pPTH-CC or pPTH-TT gave only low yields of 0.2 mg/l. Incidentally, the degenerate codons for the PTH-(1-5) region of the plasmid pPTH-TT are generally considered as " *E. coli*-favored" (Chen *et al.*, 1982). Substitution of the Ser-1 and 3 codons TCT with the other adenine-containing serine codon AGT yielded plasmid pPTH-CompB, and the PTH production was increased by 4-fold (Table 1).

Unexpectedly, transformant with plasmid pPTH-GG produced immunoreactive PTH at an even higher yield (10 mg/l, Table 1), which was later identified as a mixture of PTH-(8-84) and PTH-(1-84) with the short analogue as the major component.

For a comparative study, plasmid pPTH-hA was designed with its codons in the PTH-(1-5) region identical to those in the human cDNA (Hendy *et al.*, 1981). Expression of this plasmid generated immunoreactive PTH uncharacterized, at a yield of 0.3 mg/l (Table 1), comparable to the efficiency of plasmids inserted with the human PTH cDNA (Breyel *et al.*, 1984).

Since the utilization of A-rich degenerate codons at the PTH-(1-5) region of plasmid pPTH-AA had dramatically improved the expression, two plasmids pPTH-wA and pPTH-wxA were constructed with more A-rich degenerate codons further downstream. However, both plasmids could not generate more PTH than pPTH-AA during expression (Table 1).

Effect of E. coli-favored codon degeneracy in the mid and carboxyl-terminal regions on expression of PTH. Plasmid pPTH-AA was linearized at the *Pst*I and *Hind*III sites for the removal of its PTH-(29-84) sequence. This region was then reconstituted, with degenerate codons used in a frequency supposed to be favored by *E. coli* (Figure 2)(Chen *et al.*,1982). The new transformant JM103:pPTH-AA-Eco demonstrated a moderate increase in the efficiency of expression (7 mg/l). The new plasmid pPTH-AA-Eco was recovered to transform other bacterial strains. *E. coli* host HB101 demonstrated the same efficiency of PTH expression as in JM103, but the *lon* strain Y1091 transformant improved the yield to 20 mg/l (Table 2),. an increase of 100-fold as compared to the previously published results (Breyel *et al.*, 1984; Morelle *et al.*, 1988; Rabbani *et al.*, 1988). In addition, PTH production in strain Y1091 did not require induction by IPTG. The expressed product was eventually identified as intact PTH (described below). A prolonged growth period (16 h) significantly reduced the yield of PTH. Preliminary adenylate cyclase bioassay indicated strong bioactivity in a SDS whole cell lysate of this *lon* transformant (Rabbani *et al.*, 1988), while the lysate of a control transformant (Y1091:pUC8) failed to stimulate the formation of cAMP. Expression of PTH by plasmid pPTH-AA was also improved (6.5 mg/l) with the use of this bacterial host. All subsequent expression studies were conducted in strain Y1091.

For further studies of the effect of this "*E. coli*-like" PTH-(29-84) coding sequence on expression, the new insert was recovered through linearization at the *Pst*I and *Hind*III sites of plasmid pPTH-AA-Eco, and was used in the substitution of the existing sequences in other plasmids. Plasmid pPTH-wA-Eco, derived from pPTH-wA, expressed PTH-(1-84) at 15 mg/l (Table 2). Plasmid pPTH-hA-Eco, with the new insert, didn't produce more immunoactive PTH than its precursor plasmid pPTH-hA (Table 2). Plasmid pPTH-GG-Eco, prepared from pPTH-GG, yielded immunoreactive PTH at 25 mg/l (Table 2). Upon analysis described below, the PTH products were established to be PTH-(1-84) and PTH-(8-84), with the short analog amounting to 80-90% of the immunoreactive mixture.

The "*E. coli*-like" codon degeneracy was then extended further upstream to positions +8 and +18, and its effect on expression was investigated. Initially a precurser plasmid pPTH-(29-84)-Eco was constructed via ligation of the "*E. coli*-like" PTH-(29-84) insert with the identically linearized plasmid pUC8. The resultant plasmid pPTH-(29-84)-Eco was cut at *Eco*RI and *Pst*I sites for the subsequent construction of two plasmids pPTH-A-Eco(18-84) and pPTH-A-Eco(8-84) via the crossover linker approach already described in the synthesis of pPTH-AA. Expression of transformants with both plasmids yielded PTH-(1-84) at 7 and 14 mg/l, respectively (Table 2).

Plasmid pPTH-CompE-Eco (Table 2), with another adenine-containing degenerate codon AGC for Ser-1 and 3, produced PTH-(1-84) at 12 mg/l during expression.

Table 2. Expression of PTH genes possessing different amino-terminal nucleotide sequences in *E. coli* strain Y1091.

PTH gene -containing plasmids	PTH[a] mg/l (% protein)	N-terminal coding sequences[b] 1 2 3 4 5 Ser Val Ser Glu Ile
pPTH-AA-Eco	20 (2.5)	TCA GTA TCA GAA ATA
pPTH-wA-Eco[c]	15 (2)	
pPTH-A-Eco(18-84)[d]	14	
pPTH-A-Eco(8-84)[e]	7	
pPTH-CompE-Eco	12	AGC AGC
pPTH-GG-Eco	25[f]	G G G G
pPTH-hA-Eco	0.3[g]	T G AGT

[a] Estimated by the Allegro RIA. Values in parenthesis ( ) were percentage of bacterial protein, calculated by integrating the areas under the peak after densitometric scanning of gel.
[b] Nucleotide sequence encoding the PTH-(1-5) region of pPTH-AA-Eco is presented. For other plasmids, only nucleotides different from pPTH-AA-Eco were presented in this Table. Codon differences in other regions are stated individually.
[c] GTA-21 and TTA-24.
[d] CGT-20, 25, CTG-24 and AAA-27.
[e] AAC-10, CTG-11, 15, 24, AAA-13, 26, 27, CGT-20, GTG-21 and CGC-25.
[f] Mixture of PTH-(1-84) and PTH-(8-84). See Fig. 4.
[g] Nature of the immunoreactive PTH not determined.

Gel electrophoresis and immunoblotting. Gel electrophoresis of a lysate of clone Y1091:pPTH-AA-Eco revealed a new polypeptide with the same mobility as a synthetic sample of human PTH (Figure 3). Western blotting with antibody specific to PTH-(1-17) or PTH-(69-84) confirmed the new polypeptide as PTH-(1-84) (Figure 4). Short analogues, such as PTH-(8-84), were not detected. Exclusive production of PTH-(1-84) was also confirmed in transformants possessing plasmids pPTH-AA, pPTH-CompB, pPTH-wA-Eco (Table 1,), pPTH-A-Eco(18-84), pPTH-A-Eco(8-84), and pPTH-CompE-Eco (Table 2)(Figure 4).

Gel electrophoresis of a lysate of clone Y1091:pPTH-GG-Eco indicated mainly a new polypeptide more mobile than the synthetic human PTH (Figure 3), and it was eventually identified as PTH-(8-84). Western blotting with anti PTH-(69-84) antibodies revealed the immunoreactive product as predominently the short analogue, mixed with some PTH-(1-84) (Figure 4A). Blotting with the anti-PTH-(1-17) antibodies showed a dramatic loss of immunoreactivity of PTH-(8-84) in this lysate (Figure 4B), caused by the absence of the PTH-(1-7) region. Identitical immunoreactive mixture was also observed in transformant possessing plasmid pPTH-GG (Figure 4). Our Western blots therefore confirmed the failure of the Allegro Intact PTH RIA to exclude the PTH-(8-84) fragment in the estimation of PTH.

Extraction and purification of recombinant PTH. The acidic extraction of *E. coli* Y1091:pPTH-AA-Eco enriched PTH-(1-84) to 10% of the total protein (60% recovery) (Rabbani *et al.*,1988). The extract was processed through column chromatography on cation exchanger (55% recovery)(Figure 5A). The eluent was further purified through reverse phase chromatography (Figure 5B), to yield intact PTH as a homogeneous product. The HPLC on C_{18} silica, with its capability of separation of intact PTH, the unprocessed fMet-PTH, and analogue PTH-(8-84) in a gradient of acetonitrile in 0.1% TFA (Rabbani *et al.*,1988), revealed intact PTH as the only PTH-moiety produced. From 2 liters of culture medium, 6 mg of the recombinant intact PTH was obtained after lyophilization, with an overall recovery of 15%. Its purity was confirmed by gel electrophoresis (Figure 3), Western blots (Figure 4), and analytical HPLC on C_{18} silica in the presence of 0.1% heptafluoroacetic acid (Rabbani *et al.*,1988) or TFA.

Characterization. Amino acid composition analysis of the purified intact PTH was identical to the expected value for human PTH-(1-84) (Table 3).

Table 3. Amino acid composition of the purified recombinant intact PTH.

Amino acid	Residues/mol determined 24h	48h	72h	nearest integer	PTH-(1-84)
Asx	10.00	10.00	10.00	10[a]	10
Thr	0.82	0.82	0.80	1	1
Ser	6.08	5.46	4.90	7[b]	7
Glx	11.20	11.20	11.23	11	11
Pro	3.00	2.89	2.87	3	3
Gly	3.94	3.98	3.90	4	4
Ala	7.00	6.99	7.03	7	7
Cys	0.00			0	0
Val	7.97	8.04	7.86	8	8
Met	1.95	1.86	1.80	2	2
Ile	0.98	0.98	0.95	1	1
Leu	9.77	9.75	9.62	10	10
Tyr	0.00	0.00	0.00	0	0
Phe	0.99	1.04	1.03	1	1
His	4.04	4.09	4.06	4	4
Lys	9.07	9.04	9.01	9	9
Arg	4.95	5.03	4.97	5	5
Trp	1.06			1	1
Total				84	84

[a] 10 Asx residues/mol is assumed.
[b] Extrapolated value of 6.7 at zero time.

In repeated analyses, values of 1.95, 1.91, 2.07 and 2.09 were obtained for the number of methionine residues after hydrolysis of 24 h (Table 3), thus generally consistent to the predicted value of 2 for the processed intact PTH (Hendy *et al.*, 1981). No methionine sulfoxide has been observed in the hydrolysed residues of PTH.

Sequencing analysis of the purified intact PTH confirmed that the 40 residues at its amino-terminus was identical to that of human PTH-(1-84) (Hendy *et al.*, 1981). For sequencing other regions, the recombinant intact PTH was initially digested with endoproteinase Asp-N, which has been reported to cleave specifically at the amino-terminus of the aspartic acid residues. After separation by reverse phase HPLC (not shown), fragments were sequenced. One revealed an 11 amino acid sequence, identical to the PTH-(74-84) terminus (Hendy *et al.*, 1981). The amino acid sequence of the analogue PTH-(8-84) was also established by the same analysis.

Ionspray mass spectrum of recombinant PTH-(1-84) predominantly showed the molecular ions possessing different number of H^+ charge (Figure 6). Calculation based on different molecular ions yielded an average molecular mass of 9425.66 Da for PTH-(1-84), thus consistent to the theoretical values of 9425.26 Da. The absence of other unidentified ions generally confirmed purity of this sample.

Bioassays. The purified recombinant intact PTH was compared with synthetic samples of intact PTH and fragment PTH-(1-34) in an adenylate cyclase bioassay (Figure 7). The recombinant intact PTH stimulated adenylate cyclase in osteosarcoma cells with a k_{act} value (half maximum stimulation) of 1.6 nM, as compared to 3.8 nM for the synthetic intact PTH (Wingender *et al.*, 1989; Rabbani *et al.*,1988), and 0.59 nM for the more potent short fragment PTH-(1-34). The smaller k_{act} of the recombinant PTH, as compared to the synthetic standard, indicated a higher potency of the former.

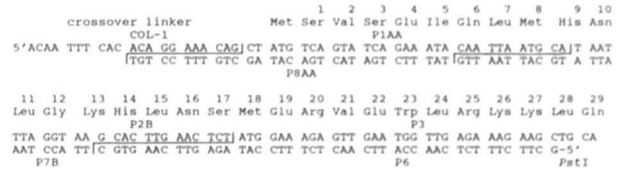

Figure 1. Oligonucleotides COL-1, P1AA, P2B, P3, P6, P7B and P8AA for the construction of the PTH-(1-28) region of plasmid pPTH-AA. After phosphorylation, the oligonucleotides were ligated to the *Pst*I end of the *EcoRI/Pst*I-cut plasmid pPTH-84. *In vivo* intramolecular recombination between crossover linker COL-1 and the *lacZ* ribosome-binding site of the plasmid yielded plasmid pPTH-AA.

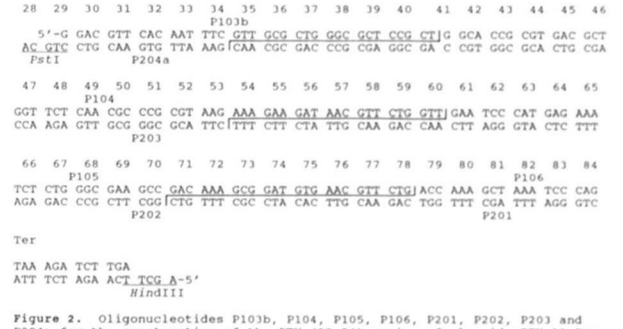

Figure 2. Oligonucleotides P103b, P104, P105, P106, P201, P202, P203 and P204a for the construction of the PTH-(29-84) region of plasmid pPTH-AA-Eco. After phosphorylation, the oligonucleotides were ligated to the *PstI/Hind*III-cut plasmid pPTH-AA to yield plasmid pPTH-AA-Eco.

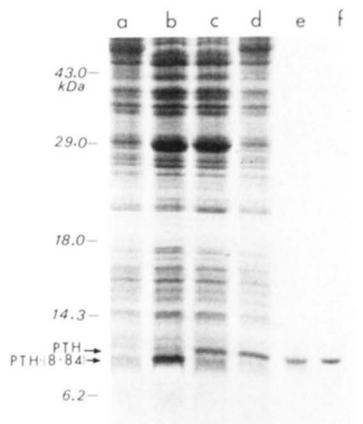

Figure 3. SDS-PAGE of PTH expressed in *E. coli*. SDS whole cell lysate (from 40 µl culture) , purified recombinant intact PTH and synthetic intact PTH (both 800ng) were electrophoresed on a 18% gel with subsequent Coomassie Blue staining. Lane a, negative control lysate of Y1091:pUC8; b, lysate of Y1091:pPTH-GG-Eco; c, lysate of Y1091:pPTH-AA-Eco; d, lysate of Y1091:pUC8 plus synthetic PTH; e, synthetic PTH; f, purified recombinant PTH. Note change of mobility of synthetic PTH when mixed with cell lysate in lane d. The positions of intact PTH, analogue PTH-(8-84) and molecular weight standards are indicated on left.

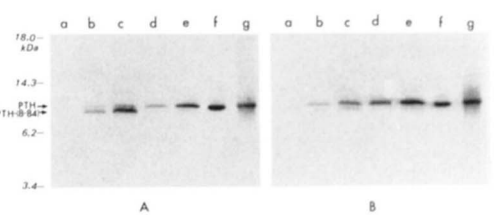

Figure 4. SDS-PAGE and immunoblotting of PTH expressed in *E. coli*. SDS whole cell lysate (from 24 µl culture), purified recombinant intact PTH, and synthetic intact PTH (both 400 ng) were electrophoresed on a 18% gel. Protein contents were electrotransfered to two nitrocellulose membranes which sandwiched the gel (200 milllamp, 15 min and then current reversed for 45 min). The membranes were immunoblotted separately with the anti PTH-(69-84) antibodies (panel A), and the anti PTH-(1-17) antibodies (panel B). Lane a, negative control lysate of JM103:pUC8; b, lysate of JM103:pPTH-GG; c, lysate of Y1091:pPTH-GG-Eco; d, lysate of JM103:pPTH-AA; e, lysate of Y1091:pPTH-AA-Eco; f, purified recombinant intact PTH; g, synthetic intact PTH. The positions of intact PTH, analogue PTH-(8-84) and molecular weight standards are indicated on left.

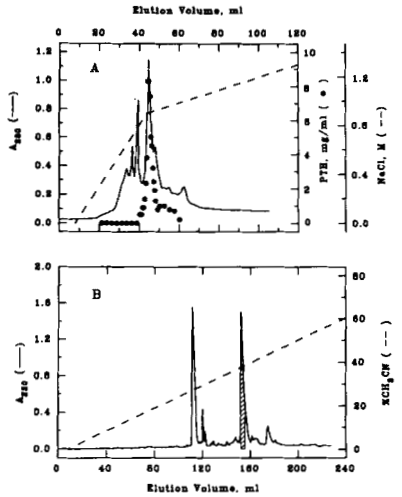

Figure 5. Purification of recombinant PTH. Panel A, chromatogram of the acidic cell extract on cation exchanger Mon S, with concentration of PTH (●) in collected fractions (1 ml) estimated. Panel B, chromatogram of subsequent HPLC purification on C_{18} silica, with PTH-containing peak (stippled) indicated.

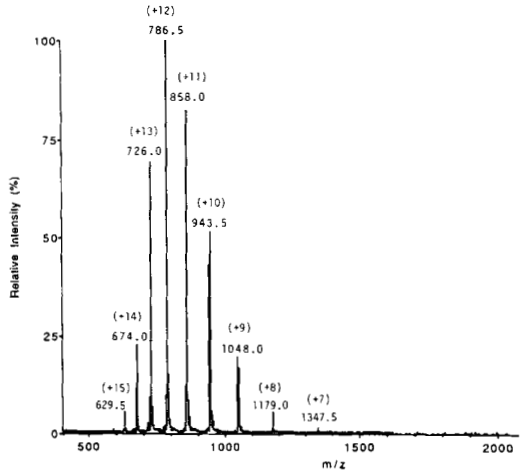

Figure 6. IonSpray mass spectrum of purified recombinant PTH-(1-84). The m/z value and z the number of H^+ charges (in parenthesis) of each molecular ion were indicated. Molecular mass is calculated by the formula of (m/z x z) - z in four most prominent peaks. Molecular mass of 9424.90, 9425.91, 9426.91 and 9424.92 Da was obtained, with an average of 9425.66 Da.

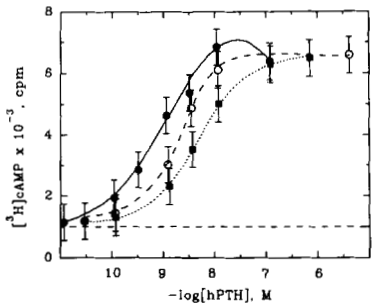

Figure 7. Adenylate cyclase assay of the recombinant intact PTH. The osteosarcoma cell (UMR 106) bioassay of purified recombinant intact PTH (○), synthetic intact PTH (■) and PTH-(1-34) (●) was carried as described.