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INTERNAL RIBOSOME-BINDING SITE DIRECTS EXPRESSION OF PARATHYROID HORMONE ANALOGUE (8-84) IN *ESCHERICHIA COLI*

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Expression of the human parathyroid hormone (PTH) gene in *E. coli* yielded intact PTH and PTH-(8-84). To determine if PTH-(8-84) is the result of a competing translation initiated from methionine codon-8 or degradation of the intact PTH, twelve new gene constructs with or without an internal ribosome-binding site (iRBS) in the PTH-(1-5) region were prepared via substitution with degenerate codons. Expression of constructs without iRBS produced only intact PTH. Constructs with weak iRBS, including one that resembles the cDNA sequence, yielded PTH-(8-84) as a minor product. In contrast, constructs with strong iRBS produced predominantly or exclusively this shorter analogue.

Human parathyroid hormone (PTH), a serum calcium regulator, is a polypeptide of 84 amino acid residues (1). Expression of a PTH gene in *E. coli* produced both intact PTH and PTH-(8-84) (2). Conceivably, the short analogue can be generated through either a proteolytic degradation of intact PTH, or an independent translation initiated from the methionine codon-8 and a weak internal ribosome-binding site (iRBS) in the PTH mRNA (3). To determine the mechanism, we have redesigned the nucleotide sequence of the PTH-(1-5) region, with or without iRBS, through substitution with degenerate codons. In expression, dramatic shifts in both the ratio of intact PTH to PTH-(8-84) and the overall expression efficiency as related to the presence of iRBS and specific degenerate codons, have been observed. Furthermore, an efficient approach has also been developed for the specific production of PTH-(8-84), an analogue which may possess novel cAMP-independent bioactivity (4, 5).

MATERIAL AND METHODS

Oligonucleotides were synthesized using a 380B DNA synthesizer (Applied Biosystem). Mass spectra were obtained by the API III LC/MS System with an ionspray interface (SCIEX, Mississauga, ONT). The amount of PTH was determined by the two-site Allegro Intact PTH radioimmunoassay (RIA) kit (San Juan Capistrano, CA). The protocol for the construction of all plasmids was

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identical to the published procedure (6). E. coli strain Y1091 (Clontech, Palo Alto, CA) was used in all expressions. Procedures for immunoblotting and the preparation of antibodies have been described (6).

RESULTS AND DISCUSSION

Twelve synthetic genes were designed, each with or without a ribosome binding site-like sequence in the PTH-(1-5) region (7), via substitution with degenerate codons without mutating the polypeptide sequence. Twelve sets of appropriate oligonucleotides encoding the PTH-(1-28) were inserted into a precursor plasmid pPTH-(29-84)-Eco to yield plasmids with different nucleotide sequence encoding the PTH-(1-5) domain (6) (Table 1). Expression of the new plasmids under the control of the *lac* promoter and operator, yielded PTH as an intracellular polypeptide.

The overall PTH expression efficiency and the ratio of PTH-(1-84) to PTH-(8-84) from 14 plasmids, including 2 previously prepared (2, 6), are tabulated (Table 1). Plasmids (#1-6) possessing no identifiable iRBS-like sequence in the N-terminal coding region produced exclusively the intact PTH as indicated by Western blotting (Figure 1). The wide range of expression efficiency suggested an order of A> G> C, T in the third base of degenerate codons immediately downstream from the starting codon.

Expression of plasmids (#7-11) designed with a weak iRBS-like sequence (7), produced a mixture of intact PTH and analogue PTH-(8-84) with the latter

Table 1. Expression of intact PTH and analogue PTH-(8-84)

#	plasmid	PTH yield[1] mg/L (1-84)	(8-84)	N-terminal coding sequence[2,3] 1 2 3 4 5 8 fmet ser val ser glu ile...met
				ATG TCA GTA TCA GAA ATA...ATG
1	pPTH-AA-Eco[4]	20		
2	pPTH-GA3-Eco	8.1		G A A A A
3	pPTH-GA10-Eco	5.9		A G A A A
4	pPTH-GA1-Eco	5.5		G G A A A
5	pPTH-CC-Eco	0.3		C C C A C
6	pPTH-TT-Eco	0.2		T T T A T
7	pPTH-GA8-Eco	4.5	1.5	A A A GAG ATA
8	pPTH-GA9-Eco	4.0	1.2	A G A GAG ATA
9	pPTH-GA4-Eco	3.5	1.5	G G A GAG ATA
10	pPTH-GA5-Eco	2.5	1	G G G GAA A
11	pPTH-hA-Eco[4,5]	0.2	0.1	T G AGT A A
12	pPTH-GA6-Eco	4	20	A A G GAG ATA
13	pPTH-GA11-Eco	1	23	T C G GAG ATA
14	pPTH-GA12-Eco		15	T T G GAG ATA

1 Total PTH was estimated by RIA and the ratio of intact PTH to PTH-(8-84) was determined by Western blotting using antibodies specific to PTH-(1-17) or PTH-(69-84).

2 Nucleotide sequence encoding the PTH-(1-5) region of pPTH-AA-Eco is presented (6). For other plasmids, only nucleotide differences are shown.

3 Potentially weak (__) and strong (__) iRBS are underlined.

4 Previously synthesized (6).

5 Ratio of the expressed products has not been reported previously.

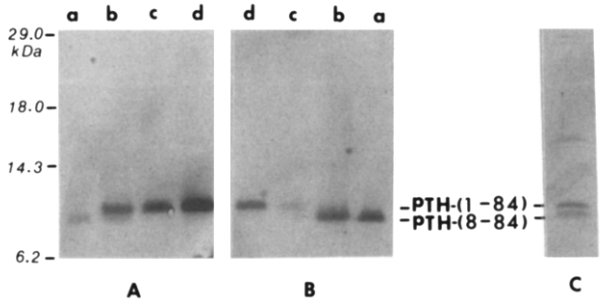

Figure 1. Immunoblotting of PTH expressed by different gene constructs in *E. coli*. SDS whole cell lysates were electrophoresed on 18% SDS-polyacrylamide gel. After electrotransfer, the nitrocellulose membrane was immunoblotted with the anti PTH-(1-17) (panel A) or anti PTH-(69-84) (panels B and C) antibodies. Panels A and B are membranes electrotransfered from the same gel. Lane a, pPTH-GA12-Eco; lane b, pPTH-GA6-Eco; lane c, pPTH-GA4-Eco; lane d, pPTH-GA3-Eco. Panel C, pPTH-hA-Eco.

as the minor component (Figure 1). Comparatively, the yields of intact PTH by the five plasmids are also consistent with the order of A> G> T-ending degenerate codons for the first two amino acid residues. Incidentally, the PTH-(1-5) coding sequence of plasmid pPTH-hA-Eco (#11), which includes a weak iRBS (1) (Table 1), is identical to that of the human PTH cDNA. Expression of this plasmid (#11) produced a 2:1 mixture of intact PTH and the analogue. Our data therefore suggested that the expression of a gene directly derived from PTH cDNA would likely produce a PTH immunoreactive mixture, instead of intact PTH previously assumed (8,9).

Expression of plasmids (#12-14), which possess a strong iRBS-like sequence (7), predominantly produced the short analogue (Figure 1). The yield of intact PTH, though greatly reduced, again depended on the selection of degenerate codons for the first two amino acid residues. Use of pyrimidine C or T-ending degenerate codons (in plasmids #13 and 14) have completely suppressed the yield of intact PTH, as compared to the case of purine A (in plasmid #12).

The exclusive expression of PTH-(8-84) by pPTH-GA12-Eco (#14) also offered a practical approach to prepare this analogue, which has been produced poorly as a by-product in the previous expression of the PTH gene (2, 3, 6). Purification of the expressed analogue involved (i) acidic extraction of culture cells of Y1091:pPTH-GA12-Eco; (ii) column chromatography on cation exchanger Mono S; and (iii) reverse phase chromatography on C_{18} silica, as in the case of intact PTH (6). Amino acid sequencing of the purified analogue confirmed that the sequence of the first 20 residues at the amino-terminus were identical to the predicted sequence. Ionspray mass spectrometry of the analogue predominantly showed the molecular ions of PTH-(8-84) charged with different number of protons H^+ (Figure 2). Calculation based on four prominent

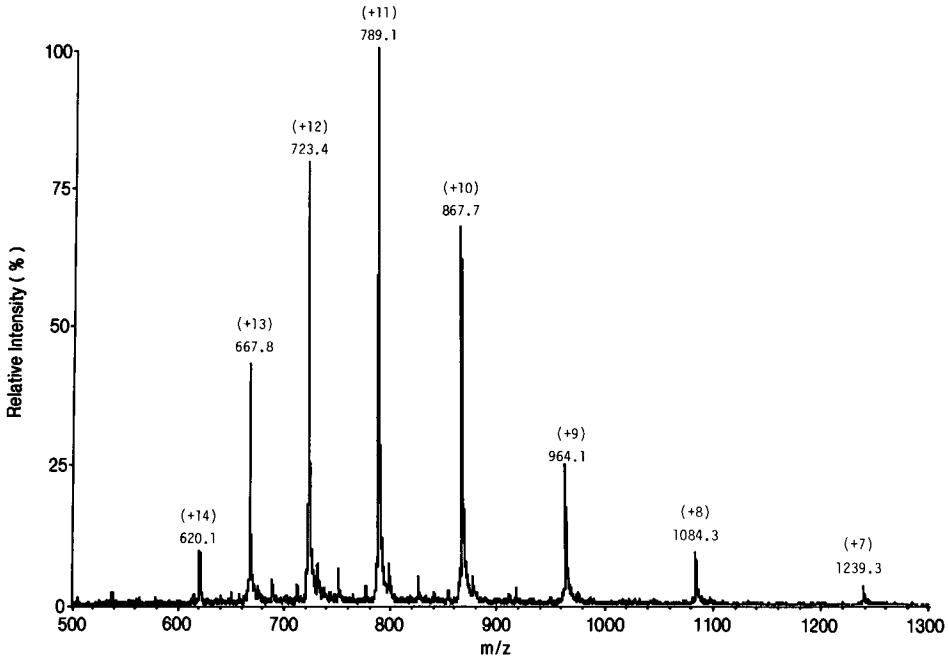

Figure 2. Ionspray mass spectrum of purified PTH-(8-84). The m/z value and z the number of H^+ charges (in parentheses) of molecular ion were given. Calculated by the formula of (m/z x z) - z, the four prominent molecular ions indicated molecular mass of 8668.30, 8668.71, 8669.01, and 8666.92 Daltons.

molecular ions yielded an average molecular mass of 8668.73 Daltons, thus consistent with the theoretical value of 8668.36 Daltons.

Further analysis of the same mass spectrum also showed traces of molecular ions of PTH-(9-84) (equivalent to 2% of the peak intensity of PTH-(8-84)). Its experimental molecular mass of 8536.08 Daltons is consistent with the predicted value of 8537.15 Daltons. For the generation of PTH-(9-84), an inefficient removal of the starting methionine-8 from the nascent polypeptide PTH-(8-84) can be predicted because of the large radius of gyration of the neighboring residue histidine-9 (10, 11). In contrast, the starting fMet residue is efficiently removed from the nascent PTH-(1-84) where the neighboring residue serine-1 has a small radius of gyration (11).

Our expression study of various PTH gene constructs in *E. coli* has now confirmed that the production of the analogue PTH-(8-84) is determined by the presence of iRBS-like sequence upstream from the codon ATG-8. Therefore, the analogue PTH-(8-84) is likely the product of an independent expression. The phenomenon of alternative translation initiation has also been observed in mammalian cells (12). In *E. coli*, such competing internal initiation may greatly surpass the normal initiation, and become the dominant pathway for gene expression under certain circumstances as demonstrated in the present studies.

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