# Nucleotide Sequences of Parathyroid Gene in Five Species of Macaque of Thailand

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The nucleotide sequences of the parathyroid hormone (PTH) gene in 5 species of macaque of Thailand, *Macaca fascicularis, M. nemestrina, M. assamensis, M. arctoides,* and *M. mulatta,* were analyzed. The sequences of 600 base pairs were determined from both strands. Single-strand DNA was prepared using streptavidin magnetic beads (Dyna beads) in combination with a biotinylated primer. The gene contains one intron which separates two exons that code the sequence of prepro-PTH and PTH. All five species of macaque showed one-hundred percent homology of the PTH gene sequence. The macaque sequences show strong homology to that of the human gene (97.4%). In addition, at the protein level, the macaque sequence has only three amino acid substitutions (at the 35th, 58th, and 76th position of PTH) as compared to that of humans. It may be concluded that this hormone is very conserved among macaques and even between macaques and humans.

**Key words:** Parathyroid hormone, macaque monkeys, nucleotide sequence.

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# ลำดับนิวคลิโอไทด์ของพาราไทรอยด์ฮอร์โมนในลิงตระกูล Macaca 5 ชนิดในประเทศไทย

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จากการศึกษาลำดับนิวคลิโอไทด์จำนวน 600 เบส ของพาราไทรอยด์ฮอร์โมน (PTH) ในลิงตระกูล Macaca 5 ชนิดในประเทศไทย จากดีเอนเอทั้งสองสาย ซึ่งดีเอนเอสายเดี่ยวเตรียมโดยใช้ streptavidin magnetic beads (Dyna beads) และ biotinylated primer พบว่าลำดับนิวคลิโอไทด์ที่ได้ประกอบด้วย intron ที่แยก exon ออกเป็น 2 ส่วนซึ่งเมื่อถอดและแปลรหัสจะได้เปปไทด์ prepro-PTH และ PTH เมื่อวิเคราะห์หาความคล้ายคลึงของลำดับนิวคลิโอไทด์ในลิงทั้ง 5 ชนิด พบว่ามีค่าเท่ากับ 100 เปอร์เซ็นต์ และเมื่อเปรียบเทียบกับลำดับนิวคลิโอไทด์ใน PTH gene ของคน พบว่ามีค่าความคล้ายสูงถึง 97.4 เปอร์เซ็นต์ และเมื่ออนุมานลำดับกรดอะมิโนจากลำดับนิวคลิโอไทด์ที่ได้และเปรียบเทียบกับลำดับกรดอะมิโนในคน พบว่ามีกรดอะมิโนเพียงสามตัวเท่านั้นที่แตกต่างกัน (ในตำแหน่งที่ 35 58 และ 76 ของ PTH) ดังนั้นจากการศึกษาในครั้งนี้สามารถสรุปได้ว่าพาราไทรอยด์ฮอร์โมนเป็นฮอร์โมนอนุรักษ์ที่ไม่มีการเปลี่ยนแปลงในระหว่างลิงตระกูล Macaca ด้วยกันและเปลี่ยนแปลงน้อยมากในระหว่างลิงตระกูล Macaca กับคน

**คำสำคัญ** พาราไทรอยด์ฮอร์โมน ลิงตระกูล Macaca ลำดับนิวคลิโอไทด์

## INTRODUCTION

Parathyroid hormone (PTH) is important in the regulation of serum calcium and phosphate levels, and both of these affect the activity of the parathyroid glands, possibly through feedback mechanisms.[1,2] PTH increases the serum calcium level by direct actions on kidney and bone, and indirect effects on gastrointestinal absorption of calcium mediated by 1,25-dihydroxycholecalciferol. PTH reduces the phosphate level by direct effects on renal excretion.[3] Furthermore, this hormone initiates bone remodeling, principally by stimulating the resorption phase. It is opposed by calcitonin of which the major action in humans, as shown by both *in vivo* and *in vitro* experiments, appears to be the direct inhibition of bone resorption. Estrogen loss, especially in aged females, limits the calcitonin level, thus throwing the balance toward an increased ratio of PTH to calcitonin.[4]

Recently, we studied the effect of estrogen injection, a popular hormonal therapy for osteoporosis, on serum PTH levels using the aged female cynomolgus macaque (*Macaca fascicularis*) as an animal model. Unfortunately, we could not succeed to measure the concentrations of macaque PTH (mPTH) using a commercial radioimmunoassay (RIA) kit for human PTH (hPTH). By these reasons, comparison of the differences of amino acid and nucleotide sequences between mPTH and hPTH from several different studies has been performed. Although there are many studies of the PTH gene in humans,[5,6] cattle,[7,8] dogs,[9] pigs,[10] and rats,[10,11] no report has yet been published on the PTH gene in monkeys. In addition, only 5 species of macaque monkeys (genus *Macaca*) occur in Thailand: *Macaca fascicularis, M. nemestrina, M. assamensis, M. arctoides,* and *M. mulatta.*[12] Accordingly, we aimed to determine the structure of the PTH gene in 5 species of macaque monkeys of Thailand to provide knowledge of the nucleotide sequence of the gene and of the deduced amino acid sequence.

## MATERIALS AND METHODS

### Animals

Five species of macaque monkeys of Thailand: *Macaca fascicularis, M. nemestrina, M. assamensis, M. arctoides,* and *M. mulatta,* were used in this study (see Table 1).

**Table 1. The habitat of each species of macaque monkeys during the blood sampling in this study.**

| Species | Habitat |
|---|---|
| *Macaca fascicularis* | Wat Kuha Phimuk, Yala |
| *Macaca nemestrina* | Chiang Mai Zoo, Chiang Mai |
| *Macaca assamensis* | Khao Laem Dam, Kanchanaburi |
| *Macaca arctoides* | Imported from Thailand to Primate Research Institute, Kyoto University, Japan |
| *Macaca mulatta* | Chiang Mai Zoo, Chiang Mai |

## BLOOD COLLECTION

A 10-ml heparinized blood sample was collected from each monkey by femoral venepuncture under anesthetization with 10 mg/kg ketamine hydrochloride (Sankyo, Japan). The buffy-coat, containing white blood cells, was separated from plasma and red blood cells by centrifugation at 2500 rpm for 10 minutes, and then used for DNA extraction.

### DNA extraction

The DNA was extracted from buffy-coat

by the method of Hashimoto *et al.* (1996)[13] using STE buffer containing 0.1 M NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA and 1% SDS.

**PCR primers for amplification and sequence determination**

Primers used for PCR amplification (446 and 448) and sequence determination (449-452) were designed according to the human PTH gene sequence[6] (Figure1 and Table 2). The nucleotide positions also follow the numbering of the human PTH sequence.[6] Primer 446 was a biotinylated primer, and primers 449, 450, 451 and 452 were FITC labeled primers (Japan Bioservice, Japan).

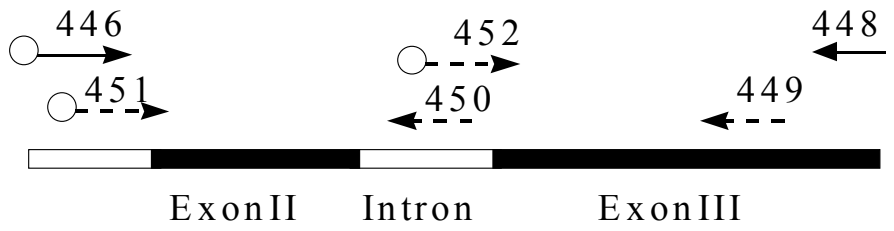


**Figure 1. The region of the monkey PTH gene and primers used for PCR amplification and sequence determination. B and F indicate the labeling with Biotin (B) and FITC (F). The direction of arrows is from 5' to 3'.**

**Table 2. The position and sequence of primers used for PCR amplification and sequence determination.**

| Number | Primer position* | Primer sequence |
|---|---|---|
| 446 | (-109)-(-90) | 5'-GCTTCTCGTGAAAACCAACC-3' |
| 448 | (496)-(516) | 5'-TTAGCAGCATGTATTGTTGCC-3' |
| 451 | (-104)-(-83) | 5'-TCGTGAAAACCAACCCAATTAG-3' |
| 449 | (473)-(492) | 5'-CACTGTCTAGAGCAGAACTC-3' |
| 450 | (157)-(176) | 5'-AAACAGAGAGGGCCACTTCC-3' |
| 452 | (166)-(186) | 5'-CCTCTCTGTTTCTCTTCTTCC-3' |

*Following the nucleotide number for humans.[6]

PCR for amplification was carried out using a thermocycler of Perkin-Elmer Cetus, model 480. The 30 thermal cycle for amplification was denaturation at 94 °C for 1 minute, annealing at 50 °C for 2 minutes, and extension at 72 °C for 3 minutes. The main cycle was preceded by heating at 94 °C for 5 minutes before the addition of *Taq* DNA polymerase. The PCR product was examined by 2% agarose gel electrophoresis.

PCR for sequence determination was carried out from single-strand DNA, namely single-strand sequencing, using a cycle sequencing kit by Applied Biosystems Inc., Japan (Applied Biosystems, model 9600).

The nucleotide sequence was determined for both strands. Sequences determined with a Shimadzu fluorescent automatic sequencer DSQ-1 were analyzed by GENETYX (Software Co., Japan).

**Preparation of single-strand DNA**

Single-strand DNA was prepared as described previously.[13] In brief, the avidin-coated magnetic beads (Dyna beads) were used in combination with a biotinylated primer. The PCR amplified products incorporated into the biotinylated primers (primer 446) were absorbed to avidin-coated magnetic beads. The single strand was eluted from the bead-bound double-strand DNA by alkalization with 0.1 N NaOH.

## RESULTS

**PCR amplification and direct sequence**

The PCR products in 5 species of macaques using primers 446 and 448 were approximately 600 base pairs. The direct sequencing revealed the presence of two exons interrupted by one intron of 103 base pairs (Figure 2). The intron followed the standard GT-AG rule at the splicing junction.[14] Comparison of whole sequence among 5 species of macaque showed 100% homology, and further comparison of the monkey gene sequences with the human gene sequence[6] indicated 97.4% homology. Furthermore, the homology between the monkey and human sequences was presented without gaps. The two exons determined in this study were identical with Exon II and Exon III of the human sequence.[6]

**Deduced amino acid sequence**

The amino acid sequence was deduced from the PTH gene (Figure 2). Exon II contained 91 base pairs that corresponds to 5 base pairs from the 5′ untranslated region, and the region that coded for the pre-sequence and the first four amino acids of the pro-sequence of prepro-PTH. Exon III contained 312 base pairs that correspond to the region coding for the remainder of the pro-sequence, PTH, and the 3′-untranslated region. An 115 amino acid polypeptide, prepro-PTH, was fully deduced. At the N-terminus, a typical 25-amino-acid signal or "pre" sequence is followed by the 6-amino-acid "pro" sequence, Lys-Ser-Val-Lys-Lys-Arg, and then by the 84-amino-acid hormone, PTH. The differences of the prepro-PTH amino acid sequence between monkeys and humans were only at the 35$^{th}$, 58$^{th}$, and 76$^{th}$ position of PTH (or at the 66$^{th}$, 89$^{th}$, and 107$^{th}$ position of prepro-PTH), changing from valine, valine, asparagine to isoleucine, isoleucine, and aspartate, respectively.[6]

## DISCUSSION

We determined the nucleotide sequence and the deduced amino acid sequence of the PTH gene in 5 species of macaque of Thailand. Our data indicate that the amplified product is approximately 600 base pairs and contains one intron and two exons. By comparing the sequences of macaque PTH and the sequence of human PTH genomic DNA, we could deduce the location of the intron interrupting the PTH gene.[6] Exon II of the mPTH gene encodes the signal sequence of monkey prepro-PTH. Such hydrophobic signal sequences are found at the $NH_2$ termini of most secreted proteins, and have an important function in the secretory process.[15] Exon III of the mPTH gene encodes the biologically active and secretes a PTH peptide of 84 amino acids. Owing to the activity of plasma endopeptidase, PTH activity may reside in numerous peptide fragments in the blood circulation, varying from 34 amino acids to 84 amino acids.[16]

From the taxonomic categories, parathyroid glands appear first in amphibians. It is not found at all in freshwater or marine teleosts, and fishes do not respond to the administration of PTH.[16,17] It seems that this hormone is highly conserved among higher vertebrates. Comparison of our monkey PTH sequences to the PTH gene sequences of the previous reports in chickens,[18] mice,[19] rats,[10] pigs,[10] cattle,[8] and humans[6] shows strong homology between macaque monkey PTH and those animal PTH sequences of 52.9%, 43.9%, 63.0%, 70.5%, 82.8%, and 97.4%, respectively. Furthermore, all five species of macaque in the present study showed the one hundred percent homology.


**Figure 2. DNA sequence and deduced amino acid sequence of the monkey PTH gene. Nucleotides in mature messenger RNA following Vasicek *et al.* (1983)[6] are capitalized; nucleotides in flanking and intervening DNA sequences are shown in small letters. A possible transcription start point is numbered 1. Star symbol (*) indicates the difference of PTH amino acids between monkeys and humans.**

Recent studies indicate three distinct and conservative regions of the PTH amino acid sequence among various species.[11] The first conservative region extends from amino acid -6 through +15, and comprises the propeptide (-6 to -1) and the $NH_2$ terminal region of PTH (+1 to +15). A second conservative region includes amino acids +23 to +38. Nussbaum *et al.* (1980) indicates that amino acids +25 to +34 are the most important for receptor binding of PTH.[20] The third area of conservation in the amino acid sequence extends from amino acid +49 to the COOH terminus of PTH at amino acid +84. By contrast, the region located between amino acids +39 and +49 shows considerable variation among rat, pig, cattle and human hormones.[11] From this approach, the PTH antibody raised from this region (amino acids +39 to +49) may represent a significant improvement in both sensitivity and specificity for PTH measurement by RIA, a competitive protein binding technique.[21] The first difference in amino acid sequence between our macaque monkeys and humans[6] is at the 35th position, when the amino acid sequence of our macaque monkeys was deduced from the PTH gene. However, determining whether changes of amino acids in this region correlate with heightened antigenicity of PTH (hormone-antibody binding) would require further study. By contrast, Usami *et al.* (1995)[22] could measure the serum mPTH level by the RIA technique using the antibody to human PTH-C (amino acids 46-84 of PTH). One may conclude that changes in the amino acid sequence at the 58th and 76th position of mPTH are unable to affect hormone-antibody binding. However, the potency of antibody binding for mPTH is less than for human PTH-C. Thus, the unsuccessful measurement of mPTH levels by the RIA technique from our previous study may have arisen from other reasons that we have not determined yet.

By comparing the location and size of the intervening DNA sequence of mPTH, completely identical in macaque and human genes, the intron is 103 base pairs long and comes between the second and third nucleotide encoding lysine-29 of the prepro-PTH molecule. On the other hand, the cattle[8] and rat[11] genes contained an intron of 119 and 111 base pairs, respectively, in the homologous location.

We found no differences in the mPTH sequence among 5 species of macaques and a small difference from those of humans, but with an identical size and location. From this we conclude that the PTH gene is very conserved among macaque monkeys and less evolved from humans.

## CONCLUSION

The direct sequencing of the PTH gene in 5 species of macaque of Thailand, *Macaca fascicularis, M. nemestrina, M. assamensis, M. arctoides,* and *M. mulatta,* has been performed. The sequence contains two exons (91 and 312 base pairs, respectively) that are interrupted by one intron of 103 base pairs. Comparison of whole sequence among 5 species of macaques shows 100% homology, and further comparison with the human gene sequence indicates 97.4% homology. The PTH gene encodes a 115 amino acid polypeptide, prepro-PTH. It consists of 25 amino acids of the "pre" sequence and 6 amino acids of the "pro" sequence of the prepro-PTH, and 84 amino acids of PTH. The differences of prepro-PTH amino acid sequence between monkeys and humans are only at the 35th, 58th, and 76th positions of PTH.

**Abbreviations:** EDTA: ethylenediaminetetraacetic acid; FITC: fluoresceinisothiocyanate; PCR: polymerase chain reaction; SDS: sodium dodecyl sulfate; Tris: trihydroxymethylaminomethane chloride.

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