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# Analysis of the Expression and the First Exon of Aromatase mRNA in Monkey Brain

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**To elucidate the mechanism of the region-specific expression of the aromatase in the primate brain, we investigated the distribution and level of the total aromatase mRNA and the aromatase mRNA with the exon 1-f, which was reported to be the brain-specific exon 1 of the human aromatase gene, in male Japanese monkeys. Total RNAs extracted from the hypothalamus-preoptic area (HPOA), amygdala (AMY), cerebellum, hippocampus, brainstem, five regions of cerebral cortex and four peripheral tissues: liver, kidney, adipose tissue and testis were subjected to a semi-quantitative reverse transcription–polymerase chain reaction–Southern blotting (RT–PCR–SB) assay. The levels of the total aromatase mRNA was high in the HPOA, AMY and testis with a low level of message in the other regions. These results roughly paralleled the distribution of aromatase activity of the monkey brain previously reported. The level of the aromatase mRNA with the exon 1-f was high in the HPOA and AMY, and low in the other regions of the brain and the testis with an undetectable level of the messenger in the other peripheral tissues. Furthermore, the ratio of the aromatase mRNA with the exon 1-f to the total aromatase mRNA was different among various regions of the monkey brain, for example, the ratio in the AMY was distinctly higher than that in the HPOA. These results indicated that the level of the aromatase mRNA mainly regulated the level of aromatase protein and aromatase activity in a region-specific manner, and that the exon 1-f was used in most of the monkey brain regions. Moreover, the ratio of the aromatase mRNA with the exon 1-f to the total aromatase mRNA varied in the brain regions. It was implied that the aromatase mRNA using the other first exons was also expressed in the brain and was involved in the region-specific expression of the brain aromatase.**

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## INTRODUCTION

The formation of estrogens from androgens is catalyzed by a specific form of cytochrome *P*450, aromatase cytochrome *P*450 ($P450_{AROM}$). This enzyme is expressed in extragonadal tissues such as the brain, adipose tissue and liver, as well as in gonadal tissues of ovary, testis and placenta in many species [1–5]. It has been well known that the levels of the enzyme in these tissues are regulated in a tissue-specific fashion [4, 6]. Previous studies demonstrated that human aromatase gene, consisting of 10 exons and spanning at least 70 kb, was isolated from the placenta [7]. Furthermore, it was clarified that the multiple untranslated first exons system was involved in the tissue-specific expression of the gene [8–15]. In the system, different exons 1 were alternatively used from the multiple exons 1 of the gene in different peripheral tissues, respectively; for example, a major transcript using exon 1-a and minor transcripts using exons 1-b, 1-c and 1-d were detected in the placenta. More recently, studies on the human brain revealed that the major transcript in the hypothalamus-preoptic area (HPOA) and amygdala (AMY) used exon 1-f, which is the "brain specific exon 1" termed by Harada *et al.* [9], and the minor transcript in the brain contained exons 1-b and I-4 [8, 12].

Aromatase in the brain plays an important role in mediating the sexual differentiation of neural structures perinatally and is involved in the regulation of reproductive behaviour in mammalian species [16–19]. Previous studies reported that aromatase activity in

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monkey brain was highest in the hypothalamus and the AMY, and was detected at lower levels in several regions of the cerebral cortex and hippocampus through the fetal, juvenile and adult stages [20–22]. To study the mechanism of these region-specific expressions of aromatase in the monkey brain, we first cloned and sequenced part of the monkey aromatase cDNA using the gene amplification method. Secondly, we investigated the distribution of the total aromatase mRNA, and the specific expression of aromatase mRNA with exon 1-f in the brain, by the use of a semi-quantitative reverse transcription–polymerase chain reaction–Southern blotting (RT–PCR–SB) assay [23]. Furthermore, we examined the ratio of aromatase mRNA with exon 1-f to the total aromatase mRNA.

## MATERIALS AND METHODS

### *Animals and tissues*

Three-year-old male Japanese monkeys *Macaca fuscata*, were used. The hypothalamus-preoptic area (HPOA), amygdala (AMY), hippocampus, frontal cortex, motor cortex, sensory cortex, temporal cortex, occipital cortex, cerebellum, brainstem, liver, kidney, adipose tissue and testis were dissected and removed from two monkeys under a deep anesthesia. The tissues were frozen in liquid nitrogen and kept at −80 C until RNA extraction.

### *Chemicals and laboratory equipment*

All reagents used in these studies were the highest grade available. Water was either double distilled and autoclaved, or treated with an additional 0.1% diethylpyrocarbonate and reautoclaved. All equipment was autoclaved or sterilized at 200 C for the elimination of RNase activity. Microtubes, pipette tips and centrifugation tubes were used only once to avoid contamination with amplified or cloned genes.

### *RNA extraction*

The total RNA was extracted from each tissue by the guanidium–cesium chloride ultracentrifugation method [24] and the RNA concentration was determined by UV absorption.

### *RT*

The total RNA extracted from each tissue was reverse transcribed to synthesize single stranded cDNA. Briefly, total RNA was incubated at 42°C for 60 min with 2 units of RAV-2 reverse transcriptase (Takara, Kyoto, Japan) in a 10 $\mu$l reaction volume containing 50 mM Tris–HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM of each dNTP and 10 $\mu$M random hexadeoxynucleotide primer (Takara).

### *Oligonucleotide primers*

The sequences of the PCR primers for the aromatase mRNAs were derived from that of the human aromatase gene because the monkey aromatase cDNA had not been cloned yet. The sequences were as follows; hAM2s: 5′-CTGAGGTCAAGGAACACAAC-3′ and hAM3as: 5′-ACCCGGTTGTAGTAGTTGCA-3′ corresponded to nucleotide residues 105–124 and 364–343 of human aromatase cDNA [7], respectively, and hAM1-fs: 5′-GAAAAGCCACCTGGTTCTTA-3′ matched sequence at the 5′-end of the brain specific exon 1-f of the human aromatase gene [9]. To analyze the total aromatase mRNA, the primers hAM2s/hAM3as were used. To analyze aromatase mRNA with exon 1-f, the primers hAM1-fs/hAM3as were used. The cDNA regions flanked by the primer sets contained one and two splicing sites, respectively. The sequences of the primers for the $\beta$-actin mRNA are as follows: $\beta$As: 5′-CCGCGAGAA-GATGACCCAG-3′, and $\beta$Aas: 5′-TGCTCCGAA-GTCCAGGGCGAC-3′. The primer set flanked the human $\beta$-actin cDNA sequence from base 426 to base 1245, as numbered by Iijima *et al.* [25], which contained one splicing site.

### *PCR*

Single stranded cDNA was subjected to PCR [26]. Briefly, 1 $\mu$l of cDNA (1/10 of obtained cDNA) was amplified in a 10 $\mu$l reaction volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 200 $\mu$M of each dNTP, 0.5 $\mu$M of each primer and 0.25 units of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). The reaction was performed for 24 cycles (to analyze the total aromatase mRNA), 28 cycles (to analyze the aromatase mRNA with exon 1-f) or 14 cycles (to analyze the $\beta$-actin mRNA) at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min with extension of the final 72°C incubation for an additional 9 min.

### *Nucleotide sequencing*

In order to confirm the authenticity of the reaction, the RT–PCR product was subjected to direct nucleotide sequencing using *Taq* DNA polymerase according to the dideoxy method [27]. To determine the nucleotide sequence near the primers, the products were subcloned into pBS M13$^+$ vector (Stratagene, LaJolla, CA. U.S.A.) and sequenced using a sequenase DNA sequencing kit (USB, Cleveland, OH, U.S.A.)

### *Southern blotting (S$\beta$)*

One microliter of the RT–PCR product (1/10 of the obtained product) from each tissue was size-fractionated by electrophoresis in a 2.0% agarose gel. After electrophoresis, the product was transferred onto a nylon membrane (Hybond N$^+$, Amersham, Bucks., England). The membrane was prehybridized in the buffer containing 6 × SSC (1 × SCC: 0.15 M sodium chloride–0.015 M sodium citrate), 150 $\mu$g/ml denatured salmon sperm DNA, 1% SDS (sodium

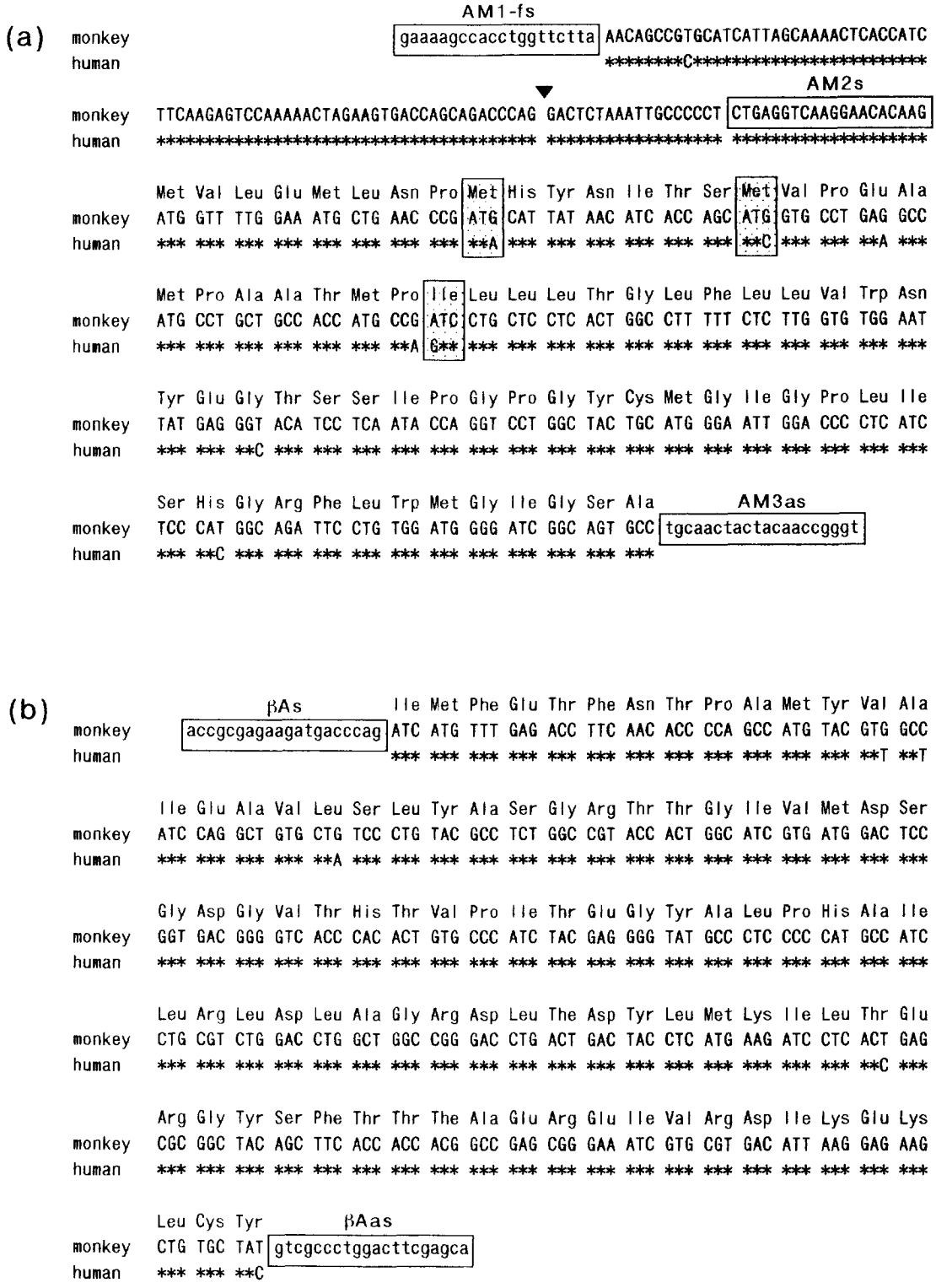


Fig. 1. Nucleotide sequence and deduced amino acid sequence of the RT–PCR products. (a) The nucleotide sequences and deduced amino acid sequence of the RT–PCR products with the primers hAM1-fs/hAM3as. The total RNA from the monkey AMY was subjected to RT–PCR with the primers, followed by nucleotide sequencing. The nucleotide sequence was 97.6% identical to the corresponding region of human cDNA, and the deduced amino acid sequence of the reading frame showed 95.9% of homology with the human homologue. The dark boxes indicate the change of amino acids; codons 9, 16 and 18 encoded Met, Met and Ile in the monkey gene instead of Ile, Ile and Val in the human one, respectively. The sequence of the product using primers AM2s/AM3as was identical to a part of that using primers AM1-fs/AM3as. * indicates the same nucleotide as the RT–PCR product. ▼ indicates the splicing site between exon 1-f and exon 2. The locations of the primers are indicated by open boxes. (b) The nucleotide sequences and deduced amino acid sequence of the products with the primers βAs/βAas. The product showed 97.7% nucleotide sequence identity and 100% homology at the amino acid level with the human homologue.

dodecyl sulfate) at 42°C for 3 h, and followed by hybridization with $^{32}$P-labeled monkey aromatase cDNA probe or monkey β-actin cDNA probe in the same buffer at 65°C for 12 h. The probes were synthesized by the random priming method using the respective RT–PCR product as a template. The membrane was rinsed twice in 2 × SSC, 1% SDS at room temperature for 10 min, followed by being washed twice in 0.1 × SSC, 1% SDS at 65°C for 20 min. The hybridization signal was analyzed by a Bioimage Analyzer, BAS-2000 (Fuji Film, Tokyo, Japan).

*Semi-quantitative comparison of the levels of the aromatase mRNAs and β-actin mRNA*

In order to compare the levels of the aromatase mRNAs and the β-actin mRNA in a semi-quantitative fashion, graded diluted total RNAs of the AMY (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 ng) were subjected to RT–PCR–SB to generate a standard curve. To analyze the level of the total aromatase mRNA, 20 ng of the total RNA from HPOA, AMY and testis, and 200 ng from the other tissues were used. To analyze that of the aromatase mRNA with exon 1-f, 20 ng of the total RNA from HPOA and AMY, and 200 ng from the other tissues were used. To analyze that of the β-actin mRNA, 20 ng of the total RNA from all the tissues were used. The levels of aromatase mRNAs and the β-actin mRNA from each tissue were calibrated using the standard curve.

*RT–PCR blank*

When distilled water as an RT–PCR blank was simultaneously subjected to RT–PCR–SB with the same reagents, no specific signal could be obtained, indicating that no contamination of any reagents occurred in these experiments.

## RESULTS AND DISCUSSION

*Partial cloning of monkey aromatase (DNA)*

Using the sequence of the previously cloned human aromatase cDNA [7, 9], three primers were designed for use in the RT–PCR. Since the primer of AM2s designed between AM1-f and AM3as, the product of the reaction with primers AM1-fs/AM3as included that with primers AM2s/AM3as.

The 390 bp of the RT–PCR product was generated from the monkey AMY using the primers AM1-fs/AM3as for the aromatase mRNA with the exon 1-f. The nucleotide sequence and the deduced amino acid sequence of the product are shown in Fig. 1(a). The nucleotide sequence of the product was 97.6% identical to the corresponding region of the human aromatase cDNA, and the deduced amino acid sequence of the reading frame of the product showed 100% of homology with the human homologue. These results indicated that the RT–PCR product was a part of monkey aromatase cDNA. Furthermore, the sequence of the product using the primers AM2s/AM3as was identical to a part of that using the primers AM1-fs/AM3as.

Using the same method, the sequence of the product for β-actin mRNA was confirmed to correspond with a part of the monkey β-actin cDNA. The nucleotide sequence and the deduced amino acid sequence of the product are shown in Fig. 1(b). The product showed 97.7% nucleotide sequence identity and 100% homology at the amino acid level with the human homologue.

*A standard curve for comparison of the levels of the mRNAs*

A standard curve was prepared from the radioactivities of the signals of the RT–PCR products generated from the graded diluted control RNAs (data not shown). Duplicate assays, which resulted in a highly reproducible response pattern, showed a linear correlation between the logarithmic value of the radioactivity of the product signals and that of the weight of the template RNA within a range of between 50 and 1.56 ng of its starting weight. Using the linear phase of a standard curve, the level of total aromatase mRNA, aromatase mRNA with 1-f and β-actin mRNA could be semi-quantified in different tissues.

*The distribution of the total monkey aromatase mRNA*

The product of 240 bp, which corresponded in length to the distance between the 5′-ends of the two primers on the human aromatase cDNA, was generated by the RT-PCR with the primers AM2s/AM3as from all regions of the brain and the peripheral tissues. The level of the total aromatase mRNA of each tissue was semi-quantified by the standard curve. The levels of the message were as follows: HPOA, AMY, testis ≫ the other tissues (Fig. 2), which roughly paralleled the distribution of aromatase activity reported previously [20–22]. This result indicated that aromatase mRNA was widely distributed in the monkey brain and peripheral tissues. Furthermore, the results suggested that the level of aromatase mRNA mainly regulated aromatase activity in a region-specific manner through the regulation of synthesis of aromatase protein in monkey brain.

The level of monkey β-actin mRNA of each tissue was also semi-quantified in order to confirm the adequacy of the RNA concentration which was determined from UV absorbance. As a result, the level of β-actin mRNA recorded in each tissue varied less than the total aromatase mRNA level and the mRNA with exon 1-f (Fig. 2), indicating that the RNA concentration could be almost accurately determined from UV absorbance.

*The distribution of monkey aromatase mRNA with exon 1-f mRNA*

Using the primers AM1-fs/AM3as, the RT–PCR products of 390 bp were generated from all regions

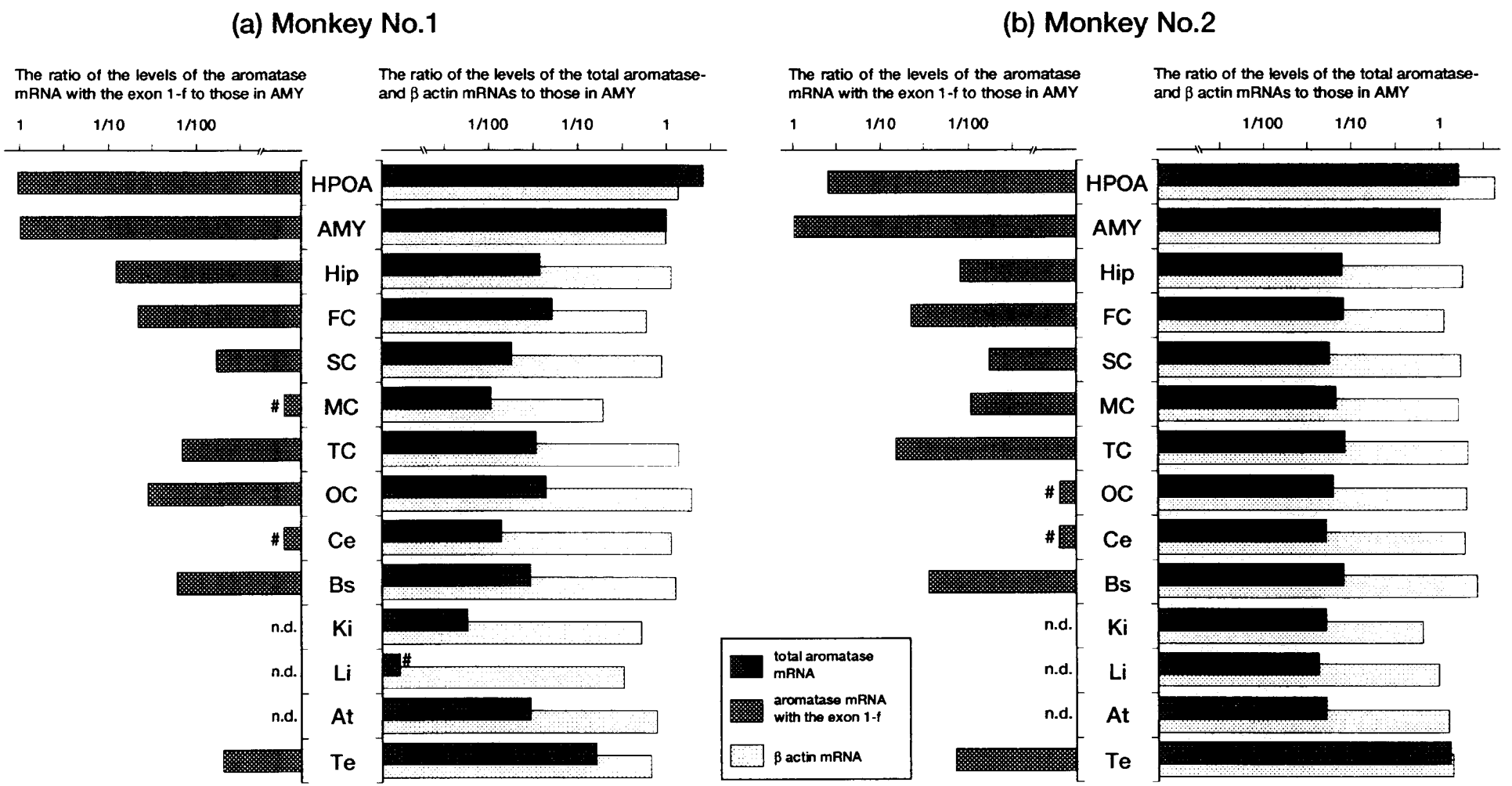


Fig. 2. The levels of total aromatase mRNA, aromatase mRNA with exon 1-f and $\beta$-actin mRNA in several regions of the brain and peripheral tissues in two Japanese monkeys, No. 1 (a) and No. 2 (b). Total RNA from each tissue was subjected to a semi-quantitative RT–PCR assay using primers AM2s/AM3as for total aromatase mRNA, primers AM1-fs/AM3as for aromatase mRNA with exon 1-f, and primers $\beta$As/$\beta$Aas for $\beta$-actin mRNA. The level of the messages in each tissue was semi-quantified using the respective standard curve. The level of mRNAs in the AMY of each monkey was assigned a value of 1.00. All other values were expressed as a ratio of this value. HPOA, hypothalamus-preoptic area; AMY, amygdala; Hip, hippocampus; FC, frontal cortex; MC, motor cortex; SC, sensory cortex; TC, temporal cortex; OC, occipital cortex; Ce, cerebellum; Bs, brainstem; Li, Liver; Ki, Kidney; At, adipose tissue; and Te, testis. # detectable but too low to quantify the level. n.d., undetectable level.

of the brain and testis. No signals of the product could be detected in the kidney, liver and adipose tissue. The levels of mRNA of each region in the brain and testis were calibrated with the standard curve except for some regions with a very low level of the message. The levels of aromatase with exon 1-f were as follows: AMY > HPOA ≫ the other regions of the brain and testis ≫ the other peripheral tissues ≒ 0 (Fig. 2). These results indicated that exon 1-f was used in most of the monkey brain regions, and that the major transcripts in the peripheral tissues used alternative first exons other than exon 1-f, the same as the expression of the aromatase gene in the human. Moreover, it should be noted that the minor transcript using exon 1-f was observed in monkey testis.

*The ratio of aromatase mRNA with exon 1-f to the total aromatase mRNA in monkey brain*

The ratio of the aromatase mRNA with the exon 1-f to the total aromatase mRNA was variable in the brain regions. It was interesting that the ratio in the AMY was distinctly higher than that in the HPOA because the hormonal regulation of the aromatase activity was reported to be different in two regions in the rodent brain [28–31]. It was reported that some minor transcripts using the other exon 1 were observed in human brain [8, 12]. Furthermore, the aromatase cDNA clone which was truncated 20 bp down-stream from the 3′-acceptor splice junction in exon 2 was detected in human fetus brain by Toda *et al.* [15]. The difference in the ratio may be due to the aromatase mRNAs using the first exons except exon 1-f or the message without the first exon. Our results together with these reports suggest that the multiple first exons system is involved in the region-specific expression and regulation of the aromatase in primate brain as well as in those in the peripheral tissues.

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