Monkeys are often used as a model system to study drug metabolism due to the similarities in drug-metabolizing properties between humans and monkeys. However, several reports indicate that species differences in the metabolism of some drugs exist (Schwartz, 2003; Gandhi et al., 2004). Among animal species used in preclinical trials, rats are commonly used to study sex differences, whereas the use of monkeys is limited, hampering our understanding of drug metabolism in this species.

In this study, we report the characterization of six novel GST cDNAs from cynomolgus monkey liver: mfGSTA1, mfGSTA2, mfGSTM1, mfMGST5, mfGSTO1, and mfGSTZ1. Five of these cDNAs have sequences highly similar to a single human GST. One particular cDNA, mfGSTA2, is highly similar to both human GSTA1 and GSTA2 cDNAs, making it difficult to determine a clear orthologous relationship for mfGSTA2 to human GSTA. Moreover, mfGSTA2 is expressed in a sex-dependent manner in liver and small intestine, unlike other identified cynomolgus GST genes.

Materials and Methods

Tissue Samples and RNA Extraction. Brain, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus tissue samples were collected from six cynomolgus monkeys (three males and three females) and used to extract RNA as described previously (Uno et al., 2006). The integrity of the RNA samples was confirmed by electrophoresis.

Sequence Analysis of Monkey GST cDNAs. Clones containing cDNAs homologous to human GSTs were selected from a large collection of expressed sequence tag (EST) clones compiled by the EST sequencing project as described previously (Uno et al., 2008). These ESTs were generated using a full-length cDNA library prepared from cynomolgus monkey liver (Uno et al., 2008). All GST clones were sequenced according to a standard protocol using the ABI PRISM BigDye Terminator version 3.0 Ready Reaction Cycle Se-
FIG. 1. Multiple alignments and phylogenetic analysis of amino acid sequences of GST cDNAs. The amino acid sequences were analyzed from human (h) and cynomolgus monkey (Macaca fascicularis; mf). A, multiple alignments of GSTA amino acids. Asterisks and dots indicate identical amino acids and conservatively unchanged amino acids, respectively. B, phylogeny of GST amino acid sequences. A phylogenetic tree was created using the PHYLIP package. Identified cynomolgus GSTs are indicated in bold letters. The scale bar represents phylogenetic distance (substitution/site).

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and the normalization of gene expression levels (Uno et al., 2006). A minimum of three amplifications was performed for each gene. For mfGSTA1 and mfGSTA2, the final concentrations of the forward and reverse primers were 50 and 100 nM, respectively. Both forward and reverse primers were used at a final concentration of 200 nM for mfGSTM5 and 100 nM for mfGSTO1, mfGSTZ1, and mfMGST1. The following primers were used: 5'-GAGGA-ACCCAAGCTCCACTCTT-3' and 5'-GTGGACATATGGGCAGAACG-3' for mfGSTA1; 5'-GAGGCCAGACGTAACAGCA-3' and 5'-CAAAGC AGTTTGGCAGTCTTA-3' for mfGSTA2; 5'-TTGGAGAACTCCTTG-GAAAAG-3' and 5'-CTGGACTCTTCTGTAGGGCAG-3' for mfGSTM5; 5'-AAGGCACAGGAAATCAGG-3' and 5'-GGATGGACACCTAGAAACAA-3' for mfGSTO1; 5'-CCTGCAGAACCCTCTGTC-3' and 5'-AGGCCCTAAATCCAGAAATG-3' for mfGSTZ1; and 5'-AGGGTTTTTCGCAATCCGAAG-3' and 5'-ATACAGGAGGCCATTTCAAG-3' for mfMGST1. To compare the levels of gene expression between the sexes, three males and three females were examined. Values were presented as mean ± S.E. Statistical significance was determined using a two-tailed unpaired Student’s t test. Values were considered significant if p was <0.05.

Results and Discussion

We have identified unique GST cDNA clones by searching our in-house EST database that was created from a full-length cDNA library prepared from cynomolgus monkey liver (Uno et al., 2008). The cDNAs for these cynomolgus GSTs, mfGSTA1, mfGSTA2, mfGSTM5, mfGSTO1, mfGSTZ1, and mfMGST1 contain an open reading frame of 222 amino acids (Fig. 1A). These GSTs were named after highly homologous human GSTs that are located in the corresponding region in the genome as described below. BLAST analysis using a putative amino acid sequence for the cynomolgus GSTs revealed a 93 to 97% sequence identity to human GSTs in the same subfamilies, reflecting an evolutionary closeness between monkey and human GST sequences. Among these, mfGSTA1, mfGSTM5, mfGSTO1, mfGSTZ1, and mfMGST1 have a high sequence identity to a single human GST, whereas mfGSTA2 cDNA is approximately 91% homologous to both human GSTA1 and GSTA2 cDNAs (Table 1). In addition, a phylogenetic comparison between the amino acid sequences of cynomolgus monkey and human GSTs (Fig. 1B) indicates that mfGSTA2 is not orthologous to any human gene. It has been reported that GSTA subfamilies arose from gene duplications that subsequently diverged in each species throughout evolution (Sheehan et al., 2001). Therefore, the orthologous relationship of GSTs between species must be determined with caution. An analysis of genome databases using the BLAT program revealed that the GSTA genes form a gene cluster in macaque and human genomes. In particular, macaque GSTA gene sequences are highly similar to the human GSTA1–5 genes with respect to their direction and position within the gene cluster (data not shown). It is noteworthy that mfGSTA2 is located at the end of the gene cluster, corresponding to the position of human GSTA2. Previous reports indicate that genes located at the end of gene clusters most likely have undergone additional recombination events, leading to the emergence of subfamily members that are less homologous to other genes within the cluster (Nelson et al., 2004). For example, this has been observed previously with cynomolgus CYP2C76 (Uno et al., 2006) and mouse CYP2C44 (Nelson et al., 2004). A further genomic sequence and protein function analysis of the identified cynomolgus GSTAs should clarify whether the function of mfGSTA2 is species-specific.

To discern whether mfGSTA2 functions differently from mfGSTA1, gene expression profiles were obtained by real-time reverse transcriptase-polymerase chain reaction using gene-specific primers and compared with the expression profiles of other identified cynomolgus GST genes. Expression patterns were examined in nine tissues, including brain, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus. mfGSTA2 is predominantly expressed in the liver with some extrahepatic expression, similar to the expression of other cynomolgus GST genes (Fig. 2A). Moreover, mfGSTA2 shows a distinct expression pattern from mfGSTA1 in these tissues, suggesting that these enzymes may play different roles in drug metabolism. Previous reports indicate that some drug-metabolizing enzymes are differentially expressed between the sexes. For example, in humans, a 2-fold increase in the expression of CYP3A4 is observed in women than in men. This coincides with the fact that CYP3A-mediated drug metabolism is more extensive in women (Wolbold et al., 2003). Therefore, gene expression profiles of mfGSTA1 and mfGSTA2 were compared between males and females in the liver and jejunum. The expression pattern of mfGSTA1 was not found to be significantly

![Table 1](attachment:image.png)

**Table 1** Amino acid identity of mfGSTA1 and mfGSTA2 compared with human GSTAs.

<table>
<thead>
<tr>
<th>GST Family</th>
<th>Human GST</th>
<th>mfGSTA1</th>
<th>mfGSTA2</th>
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<tr>
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**Fig. 2.** Expression of identified cynomolgus GST genes. The expression level of each GST gene was measured in three independent experiments and was normalized to 18S rRNA levels. A, tissue expression pattern in nine different tissues. For graphic representation, the expression level of mfGSTA1 in the liver (7.25 copies/ng total RNA) was adjusted to 1, and all other values were compared with mfGSTA1 levels in the liver. Values are expressed as the mean ± S.E. from three independent amplifications. B, expression of mfGSTA1 and mfGSTA2 for males and females in the liver and the jejunum. For graphic representation, the expression level in males was adjusted to 1 for each gene. Values are expressed as the mean ± S.E. of n = 3 animals (males or females), with the value for each animal taken as the average of three determinations. **p < 0.01.**
different between males and females (Fig. 2B). This is similar to mfGSTM5, mfGSTO1, mfGSTZ1, and mfMGST1 (data not shown).

In contrast, an 8-fold difference was observed in the jejunum of males compared with females, whereas the expression level was not significantly different in the liver between males and females (Fig. 2B). To our knowledge, a differential expression of GST genes between the sexes has not been reported in primate species. Therefore, this report provides the first evidence of a sex-dependent expression of primate GST genes. Using Western blot analysis, we detected a single protein band in the liver and jejunum of cynomolgus monkeys using human GSTA1 antibodies; however, it was not practically possible for us to distinguish mfGSTA1 and mfGSTA2 proteins probably due to their similar molecular weights (data not shown). A further investigation is necessary to determine whether mfGSTA2 protein is expressed and contributes to sex differences in drug metabolism in monkeys.

The mechanism(s) of sex-dependent gene expression has been extensively studied in rodents, especially for drug-metabolizing enzymes such as P450s and GSTs. Increasing evidence suggests that growth hormone not only regulates P450 gene expression in the liver but also controls gender-specific expression of P450 and GST genes that may account for gender differences in drug metabolism (Kamataki et al., 1984; Kato et al., 1986; Legraverend et al., 1992; Staffas et al., 1998; Park et al., 1999; Holloway et al., 2006; Waxman and O’Connor, 2006). Pulsatile release of growth hormone stimulates expression of male-specific P450 genes via activation of STAT5b, whereas a continuous secretory pattern suppresses expression of male-specific P450 genes and stimulates expression of female-specific P450 genes. Growth hormone binds to its receptor, leading to Janus kinase 2-catalyzed tyrosine phosphorylation of growth hormone receptor, which creates docking sites for STAT5b. After tyrosine phosphorylation, STAT5b dimerizes and translocates into the nucleus where STAT5b binds to the specific DNA elements to activate transcription of the target gene. This STAT5b-mediated gene transaction mechanism, at least partly, explains sex-dependent expression of some P450 and GST genes.

In humans, sex-dependent gene expression of drug-metabolizing enzymes has been observed to a far lesser extent than in rodents. For example, a 2-fold difference in the hepatic gene expression of human CYP3A4 is observed between the sexes (Wolbold et al., 2003). The investigation of sex differences in drug metabolism in humans is further complicated by individual differences such as the exposure to dietary supplements, drinking, smoking, and medication, which would influence gene expression to some extent. Because these factors can be well controlled in monkeys, this species is an ideal animal model to study gender differences with respect to human drug metabolism.

In conclusion, we have identified a novel GST gene, mfGSTA2, which shows a distinct tissue expression pattern as well as a sex-dependent expression in the liver and the jejunum, suggesting that mfGSTA2 may play a unique role as a drug-metabolizing enzyme. A further investigation of these cytochrome GSTs on protein expression levels and drug-metabolizing properties in the liver and jejunum of males and females will provide a basis for understanding species and sex differences in drug metabolism in monkeys.