Specific cDNA probes and antisera were employed to interpret genetic polymorphisms of human Mu-class glutathione S-transferases and to provide a basis for identifying individual forms in human tissues. A cDNA probe that cross-hybridized with various human and rodent Mu-glutathione S-transferase transcripts, hybridized with at least three discrete components by Northern analysis of RNA from human tissue. The smallest (1.3 kb) transcript was identified as the one that encodes GSTM3-3 subunits. A form designated GSTM5, was cloned from a human brain cDNA library and its sequence determined. The open reading frame of GSTM5 shared a high degree of homology with the sequences of other Mu-class glutathione S-transferases, but its 846-nucleotide 3′-noncoding region was unique and considerably larger than that of any of the other Mu forms. Specific synthetic peptide antigens were utilized to distinguish among and assign primary structures to individual glutathione S-transferases from human tissues. A cDNA of a new human Mu-class GST from a human brain library has been cloned, and shown to be expressed in a characteristic tissue-specific manner. The identities of other additional Mu-GSTs may be predicted from genomic Southern hybridization patterns (26) and from multiple species of the protein that have been detected in tissues. The Mu-GST counterparts in rats have been studied in detail and found to be differentially regulated in a tissue-specific manner (1). It has become a vexing problem for investigators in this field to distinguish between the multiple Mu-GSTs resolved from different human tissues and to correlate them with GSTs of known primary structure. Indeed, positive identifications of Mu-class isoenzymes have been limited. In this report the cDNA of a new human Mu-class GST from a human brain library has been cloned, and shown to be expressed in a characteristic tissue-specific manner. The identities of other individual Mu-class GSTs have been verified by use of specific antisera, and a sound molecular basis provided for studying physiological consequences of their genetic polymorphisms.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction endonucleases and most of the other enzymes and reagents as well as cloning vectors used for the molecular biology studies were obtained from Boehringer Mannheim, Promega, Sigma, or Pharmacia LKB Biotechnology Inc. Other reagents were of analytical grade. Specimens of testis, liver, and cerebral cortex were obtained from apparently normal human subjects with no evidence of pre-existing disease. The tissues obtained 3-12 h postmortem were snap frozen in liquid nitrogen and stored at -70°C.

**Methods**

**Molecular Cloning and DNA Sequence Analysis**—mRNA isolated from the frontal cortex of a 2-year-old female, was used to prepare a XZAP II cDNA library (Stratagene Inc., La Jolla, CA). Twelve 150-
mm diameter plates seeded with a total of 2.4 × 10^6 cells and were maintained by adding a 0.4-kb EcoRI-KpnI fragment of a rat Yb3 cDNA clone that cross-hybridizes with other rodent and human Mu-class GST cDNAs (27). cDNA probes used in this study were all uniformly labeled with a multiprime DNA-labeling system (Amersham) using [α-32P]dCTP (3,000 Ci per mmol) to specific activities of greater than 10^9 dpm/μg DNA. Hybridizations were performed at 42 °C in 50% formamide, 5 × Denhardt's solution, 5 × SSPE, and 0.1% SDS. Following hybridization, filters were washed with 0.1 × SSC and 0.1% SDS at 65 °C. Mu-GST-positive plaques were isolated and plaque-purified, and their DNA were isolated (28). cDNA inserts were subcloned into pBR322 plasmids (Bethesda Research Laboratories), and plasmid DNA was purified using NACS-52 PREPAC columns. Both strands were sequenced by the dideoxy chain termination method (29) using modified T7 DNA polymerase and [α-32P]dATP (United States Biochemical).

Northern Hybridizations—The following cDNA probes were used for northern blotting: 1) the 0.4-kb fragment of the rat Yb3 clone (27, 30) described above, which was used as a common probe for all Mu-GSTs; 2) a 0.3-kb HindIII-EcoRI fragment from the 3'-end of a human testis clone specific for GSTM3-3 (25); and 3) a 0.35-kb Sau3A-EcoRV fragment from the 3'-end of GSTM5 (Fig. 1). Probes were all labeled with [α-32P]dCTP as described above.

A multiple tissue Northern blot on a charge modified nylon membrane was hybridized with the 32P-labeled probes described above in 5 × SSPE, 10 × Denhardt's solution, 100 μg/ml salmon sperm DNA, 50% formamide, 2% SDS at 42 °C. The membrane was washed with 2 × SSC containing 0.05% SDS at room temperature and then at 50 °C and prepared for autoradiography.

**Fig. 1. Mu-class glutathione S-transferase transcripts in human tissues.** Northern hybridizations were with poly(A)+ RNA isolated from human tissue and transferred to a nylon membrane. Approximately 2 μg of RNA was in each lane, and the blot was tested with a 2-kb fragment of β-actin cDNA as a control. A, a cDNA probe that cross-hybridizes with many human and rat Mu-class GST transcripts (27) was labeled with [32P] and hybridized with the membrane (see "Experimental Procedures"). The autoradiography was after a 4-day exposure. B, hybridization using the same blot and a cDNA probe specific for GSTM3-3 prepared from the 3′-untranslated region of a cDNA GSTM3 that was cloned from human brain and testis expression vector libraries (25). The same blot was probed with a sequence-specific fragment from the 3′-untranslated region of GSTM3 cDNA that was cloned from human brain and testis expression vector libraries (25). The GSTM3-specific probe hybridized with the smallest of the mRNA species (about 1.3 kb), and was detected in all of the tissues with the exception of placenta. Although heart and brain were particularly rich in this form, evidently GSTM3 gene expression is not so limited as that for the brain Yt subunit in rat (30, 34). In view of evidence for the existence of additional human Mu-class GST genes (11, 23, 26, 35) and for the expression of multiple Mu forms in brain (25, 36–38), a search was undertaken for additional GSTs in human brain.

**RESULTS**

**Tissue Distribution of Mu-GST Transcripts**—A cDNA fragment that cross-hybridized with various rodent and human Mu-class GST mRNAs (27) was used to probe steady-state levels of their transcripts in eight human tissues. At least three discernible bands of mRNA in the size range of 1.3–1.6 kb were distinguished by Northern hybridization (Fig. 1). The same blot was probed with a sequence-specific fragment from the 3′-untranslated region of GSTM3 cDNA that was cloned from human brain and testis expression vector libraries (25). The GSTM3-specific probe hybridized with the smallest of the mRNA species (about 1.3 kb), and was detected in all of the tissues with the exception of placenta. Although heart and brain were particularly rich in this form, evidently GSTM3 gene expression is not so limited as that for the brain Yt subunit in rat (30, 34). In view of evidence for the existence of additional human Mu-class GST genes (11, 23, 26, 35) and for the expression of multiple Mu forms in brain (25, 36–38), a search was undertaken for additional GSTs in human brain.

**Cloning and Sequence Analysis**—A positive cloned sequence of nearly 1600 nucleotides was isolated from a human frontal cortex cDNA library. This insert, which appeared to be much larger than previously described human Mu-class GST cDNAs, also had a distinct restriction endonuclease digestion pattern. For instance, it featured a HindIII site (at position 840) and a BglII site (at position 578), neither of which were found in previously described human Mu-class GST cDNAs (11, 23–25). The complete 1557 nucleotide sequence determined for both strands of the insert, is shown in Fig. 2.

An open reading frame beginning with an ATG initiation codon consisted of 654 nucleotides encoding a protein of 217 amino acids (minus the initial methionine). The 546 nucleotides corresponding to the 3′-noncoding region of this cDNA had a polyadenylation signal sequence and is the largest 3′-noncoding region for any human Mu-class GST described thus far. An additional 57 nucleotides of the 5′-noncoding region contained a consensus translation initiation sequence (CACC) (39) immediately preceding the open reading frame. Although the nucleotide sequence in the coding region is more than 85% homologous to that of the human liver Mu-GSTs (11, 23), the 5′- and 3′-noncoding regions are largely divergent. It is therefore likely that this brain Mu-GST is the product of a separate gene.

**Primary Structures**—The deduced amino acid sequence of 217 residues, with an N-terminal proline residue and a C-terminal lysine residue, is consistent with most other human and rodent Mu-class GSTs described thus far. This protein is
and Leu'78 also distinguish GSTM4-4 from the other GSTs.

In addition, Val’69, Met’66, Lys’57, and Leu’78 also distinguish GSTM4-4 from the other GSTs. In addition, Val’69, Met’66, Lys’57, and Leu’78 also distinguish GSTM4-4 from the other GSTs.

The primary structure of GSTM5-5 is compared to sequences of other human and rodent Mu-class GSTs in Fig. 3. Only three of the first 102 residues of GSTM5-5 differ from those of human liver GSTM1-1 (“Thr”, Val16, and Arg’). Thr1 and Arg’7 are common substitutions and are found in the muscle form (GSTM2-2), but Val16 is unique among human and rodent Mu-class GSTs. In addition, Val’16, Met’15, Lys’10, and Leu’78 also distinguish GSTM4-4 from the other GSTs.

On the other hand, Lys172 is replaced by Asn and Ser in GSTM1-1 and GSTM2-2, respectively, but is found in GSTM3-3 and the rat isoforms. GSTM5-5 has five additional charged residues compared to the liver GSTM1-1 (1 Arg, 3 Lys, 3 Asp, and 2 fewer Glu residues), and on the basis of its amino acid composition, is expected to be more basic than the other human Mu-class GSTs (calculated pI of 7.1 (PGene, Intelligenetics, Inc.). There are also discrete sequence stretches invariant in all the Mu-class GSTs (Fig. 3).

Identification of Mu-class GSTs in Human Tissue—The greatest sequence divergence among the human Mu-GSTs occurs near their C termini (Fig. 3). The exon-intron organization of human Mu-GST genes is probably similar to that of the rat counterparts (35) so the divergent region is likely to be encoded by the last of the 8 exons. Accordingly, synthetic peptides corresponding to unique sequences of GSTM1-1, GSTM3-3, and GSTM5-5 (shown in brackets in Fig. 3) were selected in an effort to raise antibodies specific for each form. The sequence of GSTM2-2 is virtually identical to that of the

FIG. 2. Nucleotide and deduced amino acid sequence of GSTM5 cDNA insert. A map of the clone with the location of restriction endonuclease cleavage sites and sequencing strategies is shown below the sequence. The polyadenylation signal sequence is underlined, and the termination codon is indicated by an asterisk.

now designated GSTM5-5, based on nomenclature recently recommended for human GSTs the complete sequences of which are known (40).
GSTM1-1 peptide (one substitution of S for T), and thus the GSTM1-1 antibodies would be expected to cross-react with GSTM2-2. It is also possible that other GSTs, as yet not characterized, may cross-react with these antisera and be grouped with these classes.

Each form of the GSTs, specimens of different tissues were procured from the same individuals, and GSTs were purified by GSH-affinity chromatographic methods. SDS-polyacrylamide gel electrophoretic patterns and immunoblot analysis of matched brain, liver, and testis GSTs for three representative individuals are shown in Fig. 4. α-class GSTs were abundant in testis and liver, whereas brains were enriched in β-class forms (Fig. 4A). Mu-class GSTs were resolved into two well defined bands. Both Mu-bands cross-reacted with antisera produced against peptide antigens specific for GSTM1-1 or GSTM2-2 (Fig. 4B). GSTs from liver specimens L1 and L2 exhibited only the upper band, but no immunoreactive component at all was detected from L3. Evidently L3 in the figure represents tissue from the frequently occurring null-phenotype that was observed with 10 of 18 other liver samples (data not shown). Both brain and testis exhibited two immunoreactive bands with GSTM1-1 or -M2-2 antisera; these bands occurred in almost equivalent amounts in B1 and T1, but the lower band (of faster mobility) was a relatively minor form in testis. Only the lower band was observed for the brain and testis GSTs of the “null-phenotype” individuals (see B2 and T2).

All specimens of brain, testis, and liver, including those from individuals of Mu-null phenotype, had GSTs that cross-reacted with antisera specific for the GSTM3-3 peptide (Fig. 4C). GSTM3-3 was a prominent component in brain and testis, but only a minor form in liver (Fig. 4C). GSTM5-5 was also a major GST in brain and testis, but was not detected in liver (Fig. 4D). Both GSTM3-3 and GSTM5-5 comigrated with the GSTM1-1 band; varying ratios of the two Mu-protein bands in brain and testis indicated whether the tissues were derived from individuals of the null phenotype (see Fig. 4A).

A cDNA probe was prepared from a SauI-EcoRV fragment of the unique 3′-noncoding region of the cDNA of GSTM5 (Fig. 2). Northern hybridizations using the GSTM5-specific cDNA probe (Fig. 5A) revealed a single component primarily found in brain and lung transcripts, with lesser amounts in heart. GSTS-5 transcripts were not detected in liver, kidney, pancreas, placenta, or skeletal muscle. In contrast to multiple fragments in restriction digests of genomic DNA that hybridized with liver Mu-class cDNA probes (11, 23) the GSTM5-specific probe hybridized primarily with only a single fragment (Fig. 5B).

**DISCUSSION**

Multiple forms of mammalian GSTs are necessary to provide diversity in binding of ligands and catalytic specificities; even relatively minor forms probably have selective functions in the cell types in which they are expressed (7). Several fundamental principles concerning the human Mu-family of GSTs are implicit from cloning the GSTM5 cDNA and use of specific peptide antigens described here. Although multiple Mu forms are expressed in many tissues, tissue-specificity is determined by differences in relative amounts of each. For instance, the GST1-1 null phenotype is particularly conspicuous in liver, because it ordinarily is the primary hepatic Mu-class transcript for individuals without the gene deletion; liver is virtually devoid of GSTM5-5 and GSTM2-2 and has low levels of GSTM3-3. On the other hand, Northern blots (Figs. 1 and 5) and immunoblots (Fig. 4) show that GSTM1-1, GSTM2-2, GSTM3-3, and GSTM5-5 are all expressed in brain. Therefore, the consequence of the GSTM1 gene deletion on detoxification or protective functions associated with GSTs is likely to vary considerably in different tissues.

Northern blots probed with a cDNA fragment from the unique 3′-untranslated region, and immunoblots with the specific antisera, show that brain, testis, and lung are relatively rich in GSTM5-5. In earlier studies, the N-terminal amino acid sequence of a Mu-class GST of brain (named GST6) (38) was found to be identical to that of the GSTM2-2 of muscle, but its isoelectric point as well as electrophoretic and immunological properties differed so that its identification was uncertain. In lung two distinct Mu-class GST subunits were resolved by SDS-polyacrylamide gel electrophoresis (41); one of these did not show genetic polymorphism (42). Even though those GSTs were not assigned primary structures, it is likely that GSTM5-5 is a prominent pulmonary Mu form and GSTM3-3 is expressed in lung as well. The tissue distribution of GSTM5-5, however, appears to be more limited than that of the other human GSTs.

The GSTM2 gene was originally cloned from muscle but common reference to this form as the “muscle enzyme” evidently is a misnomer, since GSTM2-2 is a major Mu-subunit in brain and other extrahepatic tissues. Peptide-specific antisera (Fig. 4) may be used to distinguish between GSTM2-2 and GSTM1-1 on the basis of differences in electrophoretic mobilities, even though there is extensive sequence homology of both proteins and of their mRNAs (including the 3′-nontranslated region) (24). Moreover, the presence of GSTM2-2 in brain, testis (Fig. 4) and other extrahepatic tissues of individuals lacking the major liver Mu form, confirms the supposition that GSTM1 rather than its GSTM2
**Human Mu-Glutathione Transferases**

**Identification of Mu-class GSTs**

GSTs were purified from tissues of three individuals and resolved by SDS-polyacrylamide gel electrophoresis. L₁, B₁, and T₁ represent total GSTs purified from liver, brain, and testis of the same individual. Approximately 5 μg of protein was applied to each lane and the indicated electrophoretic mobilities of Alpha, Mu, and Pi forms were resolved on the basis of differences in isoelectric points, chromatographic elution patterns, and electrophoretic mobilities, it is often difficult to assign individual components to GSTs of known primary structures. For instance, only two Mu-class subunits were detected by HPLC analysis of testicular GSTs (43); the current study suggests that at least four are expressed in testis. Chromatofocusing methods have been used to resolve five Mu-GSTs from heart and sorta (44) and multiple forms from skeletal muscle (45), but assignments of those forms to GSTs of known primary structures remained uncertain. In that respect N-terminal sequence analyses frequently reported for Mu-isoenzymes that are not blocked, are inadequate because of largely invariant sequences in this region (see Fig. 3). However, use of the peptide-specific antisera described here, combined with inherent differences in electrophoretic mobilities on SDS gels alone, permit identification of four of these four GSTS (Fig. 3) in any tissue.

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**FIG. 4. Identification of Mu-class GSTs in liver, brain, and testis.** GSTs were purified from tissues of three individuals and resolved by SDS-polyacrylamide gel electrophoresis. L₁, B₁, and T₁ represent total GSTs purified from liver, brain, and testis of the same individual. Approximately 5 μg of protein was applied to each lane and the indicated electrophoretic mobilities of Alpha, Mu, and Pi forms were determined using specific antisera for each class (data not shown). A, a Coomassie Blue-stained gel; B, immunoblot of the same GST samples with antisera produced using the GSTM1-1 peptide antigen (see Fig. 3); C, immunoblot with GSTM3-3 specific antisera; and D, immunoblot using GSTM5-5-specific antisera.

**FIG. 5. Tissue distribution of GSTM3-5.** A, Northern hybridization of the blot described in Fig. 1 using a SnaI-EcoRV fragment from the 3'-untranslated region of the cDNA (Fig. 2) as a probe. Autoradiography was after a 10-day exposure. Labels are the same as those in Fig. 1. B, Southern hybridizations with the same probe with restriction digest fragments of human genomic DNA. The DNA was digested with BglII (B), PstI (P), HindIII (H), and EcoRI (E).
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