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Genetics

Hereditary differences in the expression of the human glutathione transferase active on *trans*-stilbene oxide are due to a gene deletion

(mRNA expression/cDNA clone/DNA polymorphism)

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ABSTRACT **Glutathione transferase (GT; EC 2.5.1.18) mRNA levels were measured in human liver samples by using mouse and human cDNA clones that encode class-mu and class-alpha GT. Although all the RNA samples examined contained class-alpha GT mRNA, class-mu GT mRNA was found only in individuals whose peripheral leukocytes expressed GT activity on the substrate *trans*-stilbene oxide. The mouse class-mu cDNA clone was used to identify a human class-mu GT cDNA clone, λGTH411. The amino acid sequence of the GT encoded by λGTH411 is identical with the 23 residues determined for the human liver GT-μ isoenzyme and shares 76–81% identity with mouse and rat class-mu GT isoenzymes. The mouse and human class-mu GT cDNA inserts hybridize with multiple *Bam*HI and *Eco*RI restriction fragments in the human genome. One of these hybridizing fragments is missing in the DNA of individuals who lack GT activity on *trans*-stilbene oxide. Hybridizations with nonoverlapping subfragments of λGTH411 suggest that there are at least three class-mu genes in the human genome. One of these genes appears to be deleted in individuals lacking GT activity on *trans*-stilbene oxide.**

The glutathione transferases (GTs, EC 2.5.1.18) are a family of catalytic and binding proteins that detoxify chemical carcinogens (1, 2). Multiple GT isoenzymes have been isolated from human, rat, and mouse tissues (2). Protein sequence data and antibody cross-reactivity have been used to group these isoenzymes into three distinct classes that have been termed alpha, mu, and pi (3). Members of the same class share 75–95% amino acid sequence identity, whereas members of different classes share 25–30% sequence identity. Livers of all adult humans express several class-alpha GTs; in addition, the livers of about one-half of the adult population contain a class-mu isoenzyme, GT-μ (3, 4). The GT-μ isoenzyme has been shown to be similar or identical to a GT activity against *trans*-stilbene oxide (GT-tSBO) that is measured in peripheral leukocytes (5). Studies on several hundred subjects have shown a large variation in GT-tSBO activity among individuals; ≈50% of the population lacks GT-tSBO activity (6). Individuals lacking GT-tSBO are more likely to contract lung cancer (7).

Three genetic loci encoding human liver GT isoenzymes have been characterized: *GST1*, *GST2*, and *GST3* (8–10). *GST1* corresponds to the class-mu isoenzyme, *GST2* corresponds to the class-alpha isoenzyme, and *GST3* encodes the placental class-pi GT (3, 11). The *GST1* locus is polymorphic in human populations and displays three alleles: *GST1-0* (null), *GST1-1*, and *GST1-2* (8–10, 12). Studies on Indian, Chinese, and Caucasian populations have shown that the fraction of the population with two null *GST1* alleles ranges from 31% to 66% (8, 12). The polymorphism at the *GST1* locus is similar to the variation in GT-tSBO activity and is consistent with the observation that the *GST1* locus encodes the GT-μ isoenzyme (J.S., unpublished data) that is responsible for GT-tSBO activity (5). Three additional human loci have been described: *GST4*, *GST5*, and *GST6* (12, 13). *GST5*, which is expressed in brain, cross-reacts with antibodies raised against *GST1* and thus is likely to be a class-mu isoenzyme; *GST4* and *GST6* were not precipitated by antibodies raised against *GST1*, *GST2*, or *GST3* (13) and thus cannot be placed in a GT class.

In this paper, we show that individuals who lack GT-tSBO activity do not express class-mu GT mRNA in the liver. We have isolated and sequenced a human class-mu cDNA clone[§] that encodes a polypeptide with an amino-terminal sequence identical to the human GT-μ enzyme. We also show that low GT-tSBO activity is associated with the absence of *Bam*HI and *Eco*RI restriction fragments in genomic DNA. Our results suggest that the *GST1-0* (null) genotype is due to the deletion of a class-mu GT gene.

MATERIALS AND METHODS

Nomenclature. References to the rat GT isoenzymes follow the guidelines suggested by Jakoby *et al.* (14). References to the mouse isoenzymes follow Pearson *et al.* (15, 16). In this paper, we refer to the three classes of mammalian soluble GTs as alpha, mu, and pi; we refer to the class-mu human liver GT isoenzyme measured with the substrate 1-chloro-2-4-dinitrobenzene as GT-μ (3). We refer to the human genetic loci that encode GTs as *GST1*, *GST2*, and *GST3* (8, 9).

Reagents. [^{32}P]dCTP was purchased from New England Nuclear or ICN Radiochemicals (Irvine, CA). Enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, and Boehringer Mannheim. The murine liver GT cDNA plasmids pGT875 (GT class-mu) and pGT41 (class-alpha) have been described (16). Eighteen of the amino-terminal 23 amino acids determined for human GT-μ (3) are identical to those encoded by pGT875 (78% identity). pGT41 shares 78% DNA sequence identity and 76% amino acid sequence identity with the human class-alpha GT cDNA clones pGTH1 (17, 18) and λGST2-3 (19).

Methods. Human liver biopsies and blood were obtained from kidney donors and patients scheduled for gall bladder surgery. Liver and peripheral blood was also obtained from a fetus that aborted in the 16th week of gestation. Horizontal starch gel electrophoresis was used to determine the *GST1* phenotype (8). GT-tSBO was measured in whole blood or isolated leukocytes to discriminate between the homozygous low (GT-tSBO activities <0.26 nmol of GT-tSBO per min per ml of whole blood and <0.06 nmol of GT-tSBO per min per

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Abbreviations: GT, glutathione transferase; GT-tSBO, GT activity on the substrate *trans*-stilbene oxide; nt, nucleotides.

[§]The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03817).

mg of protein in liver cytosol), heterozygous high (0.40–0.75 nmol per min per ml of whole blood, 37–63 nmol per min per mg of protein in liver), and homozygous high (1.15–1.70 nmol per min per ml of whole blood) phenotypes (5, 6, 20, 21).

Total cellular RNA was purified from human liver tissue samples by guanidinium isothiocyanate homogenization followed by CsCl centrifugation (22). RNA was electrophoresed and transferred to nylon membranes as described (16). Human DNA was purified from leukocytes, digested for Southern blot analysis, electrophoresed, and transferred to nylon membranes (23). Hybridizations of RNA and DNA transfers with murine cDNA probes were incubated in a solution of 0.5 M sodium phosphate buffer (pH 7), 7% (wt/vol) $NaDodSO_4$, 1% bovine serum albumin, and 1 mM Na_2EDTA, at 60°C, and were washed in 40 mM sodium phosphate buffer at 50°C (24). Hybridizations with the human λGTH411 probe were incubated and washed at 65°C.

cDNA Cloning and Sequencing. A human liver cDNA library from an individual expressing GT-tSBO (Fig. 1, lane d) was constructed in the phage vector λgt10 (25). The library was screened by hybridization with ^{32}P-labeled pGT875 insert at 60°C in 1 M NaCl that was followed by washing at 50°C in 50 mM NaCl as described (23). The DNA sequence of the λGTH411 insert was determined by dideoxy chain-termination techniques by using Sequenase (United States Biochemical, Cleveland). The sequence was determined on both strands; each nucleotide of the GTH411 insert was determined an average of seven times.

RESULTS

Expression of Class-Mu and -Alpha GT mRNAs in Human Liver. There is substantial evidence that the human GT-μ enzyme is responsible for GT-tSBO enzyme activity measured in peripheral leukocytes (5) and that this enzyme is encoded at the *GST1* locus (J.S., unpublished data). In about 50% of the population, both of the parental *GST1* alleles are null. To determine whether this lack of GT-tSBO activity is due to a lack of GT-μ mRNA, we hybridized a mouse class-mu GT cDNA clone (pGT875) to preparations of total human liver RNA from individuals whose GT-tSBO activity had been determined (Fig. 1*A*). pGT875 encodes the class-mu mouse liver GT enzyme GT8.7 (15). In these experiments, pGT875 hybridized with a discrete species of liver mRNA from every liver tissue sample that contained liver GT-tSBO activity (hybridization with the RNA in lane f, which is very faint in *A*, can be seen more clearly in *C*). In samples obtained from individuals lacking this enzyme activity, no GT-specific hybridization was observed. Lane e contains RNA from a fetal liver displaying homozygous high GT-tSBO levels in peripheral blood (1.20 nmol per min per ml of whole blood) but low activity in the liver (4.0 nmol per min per mg of protein). We did not detect any class-mu GT mRNA by using the mouse cDNA probe from this sample. Thus levels of class-mu mRNA and GT-tSBO enzyme activity in liver show a strict correspondence.

In contrast, class-alpha GT mRNA was detected in each of the liver tissue samples, regardless of the levels of GT-tSBO expression, when these RNA samples were probed with the mouse class-alpha cDNA clone pGT41 (16) (Fig. 1*B*). Two mRNA species hybridized with the class-alpha GT probe; both are smaller than class-mu GT mRNA. Since class-alpha isoenzymes are homo- and heterodimers in humans and rats, the two mRNA species may correspond to the Ha and Hc human class-alpha subunit mRNAs (17).

Characterization of the Human Class-Mu GT cDNA Clone λGTH411. We have isolated a human liver class-mu GT cDNA clone from a λgt10 phage cDNA library constructed from poly(A)$^+$ mRNA obtained from the sample shown in Fig. 1, lane d. The individual from whom the liver was

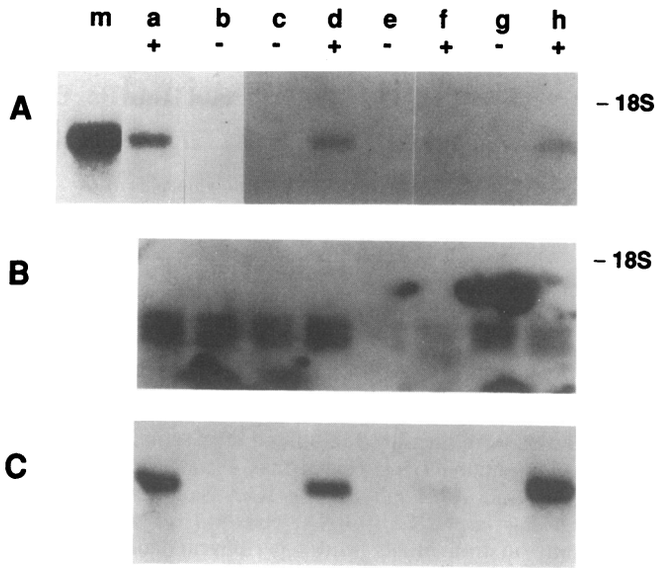

FIG. 1. Expression of GT mRNA in human liver. Nylon membranes containing human liver RNA samples were hybridized with ^{32}P-labeled murine and human GT cDNA clones. Each of the panels contains the same set of adult (lanes a–d and f–h, 10 μg) or fetal (lane e, 10 μg) human total liver RNA samples. (*A*) Hybridization with pGT875, a cDNA clone that encodes the mouse class-mu GT GT8.7. Lane m contains total CD-1 female mouse liver cellular RNA (2 μg). (*B*) Hybridization with pGT41, a mouse cDNA clone that encodes class-alpha GT isoenzyme (15). (*C*) Hybridization with the human class-mu cDNA insert in λGTH411. The level of GT-tSBO activity in the liver samples is indicated as positive (+) or negative (−). The levels of GT-tSBO activity in the blood for the individuals in lanes a–h were (nmol per min per ml of whole blood) as follows. Lanes: a, 0.58; b, 0.11; c, 0.09; d, 0.65; e, 1.20 (fetal); f, 0.81; g, 0.09; h, 0.68. Liver GT-tSBO activities for the samples in lanes a and b were not determined but are inferred from the peripheral blood GT-tSBO activities. Liver GT-tSBO activities for the samples in lanes c–h were (nmol per min per mg of protein) as follows. Lanes: c, 0.0025; d, 59.0; e, 4.0; f, 30.4; g, 0.0023; h, 43.0. The larger band seen in *A*, lanes a and b, is due to hybridization of the plasmid vector with human 18S RNA. Lanes c–h of *A* and lanes a–h of *B* were probed with a random-primer-labeled plasmid insert.

isolated expressed the *GST1-1* phenotype, as determined by starch gel electrophoresis (8). Two hundred thousand plaques in the human liver library were hybridized with ^{32}P-labeled pGT875 insert and a single cross-hybridizing plaque, λGTH-411, was isolated and purified, and its DNA sequence was determined. λGTH411 contains an 1100-base-pair insert that shares 78% nucleotide sequence identity with the first 801 nucleotides (nt) of the mouse pGT875 probe (16). The GTH411 insert encodes a polypeptide of 217 amino acids (M_r, 25,552) that shares 76–81% amino acid sequence identity with murine and rat class-mu GT isoenzymes (Fig. 2*B*). The predicted 23 amino-terminal residues of the human GTH411 insert are identical to those determined for the human GT-μ isoenzyme (Fig. 2*B*) (3).

The human class-mu cDNA insert in λGTH411 is only distantly related to the class-mu and class-pi isoenzymes. Human GTH411 shares only 27% amino acid sequence identity and 44% nucleotide sequence identity with the human class-alpha GT cDNA clones, pGTH1 (17, 18) and λGST2-3, respectively (19) (alignments not shown). GTH411 shares 30% protein sequence identity with the rat7-7 class-pi GT isoenzyme (27).

The GTH411 insert was also used to measure human class-mu GT mRNA levels in liver samples (Fig. 1*C*). These hybridizations were performed under more stringent conditions (65°C incubation and wash), but the pattern of expression was identical to that seen with the mouse class-mu

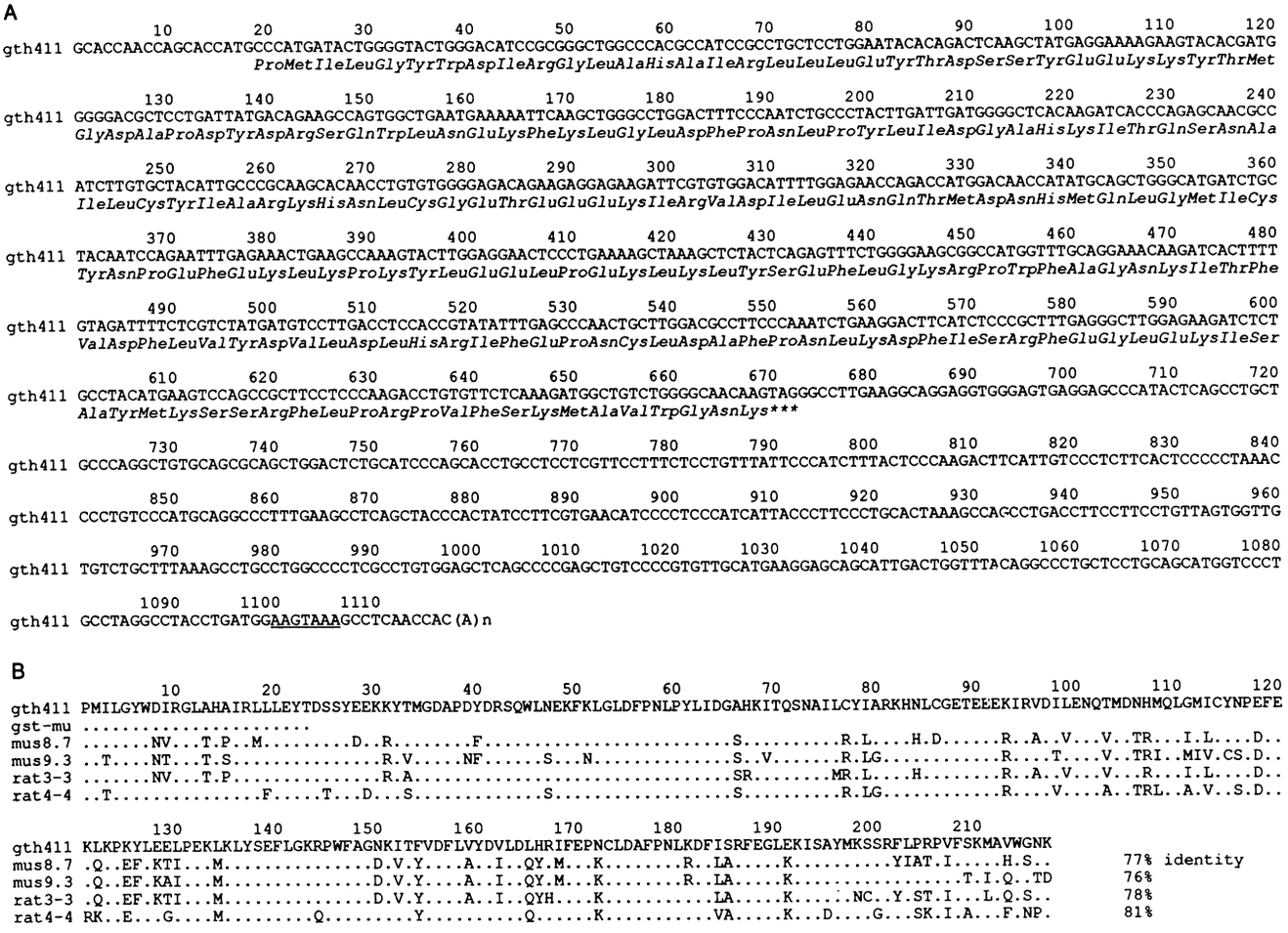

FIG. 2. Nucleotide and amino acid sequence of the human class-mu GT cDNA clone λGTH411. (*A*) The nucleotide and amino acid sequences of the GTH411 insert. The termination codon is indicated (★★★) and a potential poly(A) addition site (AAGUAAA) is underlined. (*B*) Comparison of the GT subunit encoded by GTH411 with human GT-μ (gst-mu), and mouse and rat class-mu GT isoenzyme sequences. Only amino acid replacements with respect to GTH411 are noted. The mouse GT8.7 (mus8.7) and GT9.3 (mus9.3) sequences were determined by Pearson *et al.* (16). The rat3-3 and rat4-4 sequences were determined by Pickett and coworkers (26).

cDNA probe. No hybridization was observed in liver RNA samples from individuals lacking GT-tSBO.

Southern Blot Analysis of Human Class-Mu GT Genes. To determine the genetic basis for the absence of class-mu mRNA in human livers that lack GT-tSBO activity, we hybridized the mouse and human class-mu cDNA probes to Southern transfers containing restriction digests of human genomic DNA. DNA samples from subjects with known GT-tSBO phenotypes were digested with the restriction enzyme *Bam*HI and probed with the murine pGT875 cDNA insert (Fig. 3*A*). Alternatively, human DNA samples were digested with *Eco*RI and probed with the human GTH411 insert (Fig. 3*B*). When the mouse class-mu probe pGT875 was used, six *Bam*HI fragments ranging from 6 kilobases (kb) to 17 kb were observed in each of the human DNA digests. However, a seventh *Bam*HI fragment at 11.5 kb was found in individuals expressing GT-tSBO. *Bam*HI patterns of class-mu GT hybridization from the *GST1-1* and *GST1-2* genotypes were indistinguishable. An analysis of DNA from 25 individuals showed that the 13 samples with high GT-tSBO activity contained the 11.5-kb fragment, whereas the 12 with low GT-tSBO activity lacked this fragment. When the human GTH411 cDNA insert was used to probe *Eco*RI digests of human DNA, eight fragments ranging in size from 1.9 kb to 20 kb were seen, and an additional 8-kb *Eco*RI fragment was found in individuals expressing GT-tSBO.

Multiple bands on a Southern blot of genomic DNA, such as those shown in Fig. 3, are sometimes due to multiple genes, but they can also be due to multiple exons on different restriction fragments. The 11.5-kb *Bam*HI band and the 8-kb *Eco*RI band absent in individuals lacking GT-tSBO activity could, therefore, be due to the loss of all, or a portion, of a class-mu GT gene. To estimate the number of human class-mu GT genes, we compared the hybridization patterns found with the complete GTH411 insert to those obtained with either the 5′ or 3′ half of the insert (Fig. 4). Restriction fragments that contain a complete gene should hybridize with both halves of the cDNA insert. If a restriction fragment hybridizes with one half of the cDNA but not the other, it contains only a portion of the gene.

Comparison of lane a with lanes c and e in Fig. 4*A* shows that there are two *Eco*RI bands that hybridize with both the 5′ half and the 3′ half of the GTH411 cDNA insert. Thus, there are at least two other class-mu GT genes in addition to the gene that is associated with GT-tSBO activity and the 8-kb *Eco*RI fragment. In the *Bgl* II digests, none of the bands that hybridize with the full-length probe hybridizes with both the 5′ half and the 3′ half. This is expected for the gene that encodes the sequence in GTH411, since the 5′ and 3′ subclones were constructed by cleavage at a *Bgl* II restriction site in the cDNA. However, since none of the *Bgl* II bands hybridizes with both halves of the GTH411 probe, it appears that all of the class-mu genes seen in Fig. 4 share the *Bgl* II site found in the GTH411 insert. In the *Bam*HI digests of human DNA, there are several bands that hybridize with both the 5′ and 3′ probes, which suggests that there are at least two

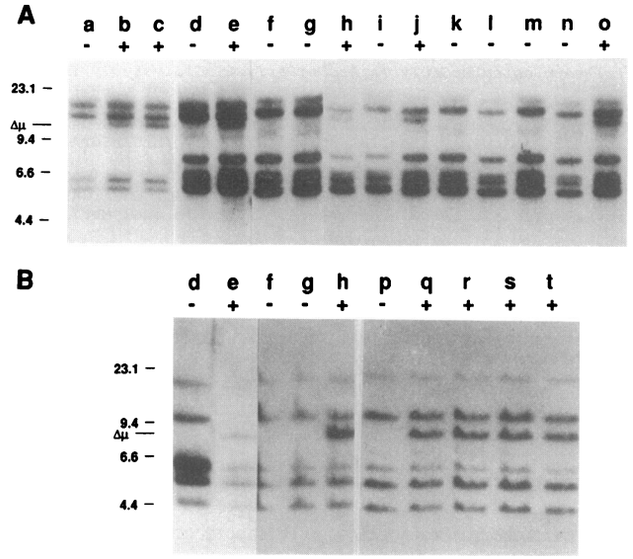

FIG. 3. Southern blot analysis of human DNA with pGT875 (mouse class-mu GT). DNA samples (5–15 μg per lane) from individuals who had been assayed for leukocyte GT-tSBO activity were digested with *Bam*HI or *Eco*RI, electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane. Levels of GT-tSBO activity measured in peripheral blood are indicated as positive (+) or negative (−). DNA samples from 20 unrelated individuals are shown; the samples in lanes d–h are the same in *A* and *B*. (*A*) Human DNA samples were digested with *Bam*HI and hybridized with a mouse class-mu cDNA insert (nt 1–715 of pGT875). The position of an additional 11.5-kb *Bam*HI band that is present in DNA from individuals with high GT-tSBO activity is shown (Δμ). The positions of the λ*Hin*dIII DNA size standards are also indicated. (*B*) Human DNA were digested with *Eco*RI and hybridized with the human class-mu GT cDNA insert from λGTH411. The position of an 8-kb *Eco*RI band found in individuals expressing GT-tSBO in peripheral blood is indicated.

class-mu GT genes present in the DNA of individuals who do not express GT-tSBO.

The detection of multiple bands that hybridize with the class-mu probe is not due to polymorphism in the human population. Each of the 25 DNA samples that we examined displayed exactly the same pattern of *Bam*HI fragments, with the exception of the 11.5-kb fragment associated with GT-tSBO activity. Likewise, each of the 20 samples examined with the GTH411 probe showed the same *Eco*RI pattern, except for the 8-kb fragment. (Some minor differences seen in Fig. 3 are due to differences in the extent of restriction enzyme digestion and electrophoresis conditions.)

Our results suggest that the restriction fragments missing from individuals lacking GT-tSBO activity are missing because of a gene deletion. To test this hypothesis, we measured the amount of hybridization to lanes a and b of Fig. 4 *A–C*. If a gene deletion has occurred, there should be more total hybridization to the GTH411 insert in the high GT-tSBO samples. Densitometry of lanes a and b of Fig. 4 confirms that the amount of hybridization in lane b (high GT-tSBO) is 25–31% higher than the level in lane a. In this measurement, we have used the internal standards provided by bands that are shared between individuals with high and low GT-tSBO activities. For example, the optical density of the 4.4-kb *Eco*RI band in Fig. 4*A* is 0.67 in lane a and 0.70 in lane b, and the sum of the optical densities of all the peaks shared in lanes a and b is 3.71 in lane a and 3.64 in lane b. However, an additional peak with an optical density of 1.13 (31% of 3.64) is seen at 8 kb in lane b. In some digests (Fig. 4*B*, lanes b and f and Fig. 4*C*, lanes b, d, and f), this additional hybridization is seen in a band that migrates the same distance as bands present in DNA from the individual with low GT-tSBO activity. The decreased amount of hybridization to the DNA of individuals lacking GT-tSBO activity, therefore, suggests that the differences in restriction patterns seen in Figs. 3 and 4 are due to a gene deletion.

DISCUSSION

By using mouse and human class-mu cDNA probes, we have shown that class-mu mRNA is absent from the livers of humans who lack GT-tSBO. In contrast, class-alpha mRNA was found in all of the livers tested, regardless of whether the individual expressed GT-tSBO activity. The perfect correlation between class-mu mRNA hybridization and high GT-

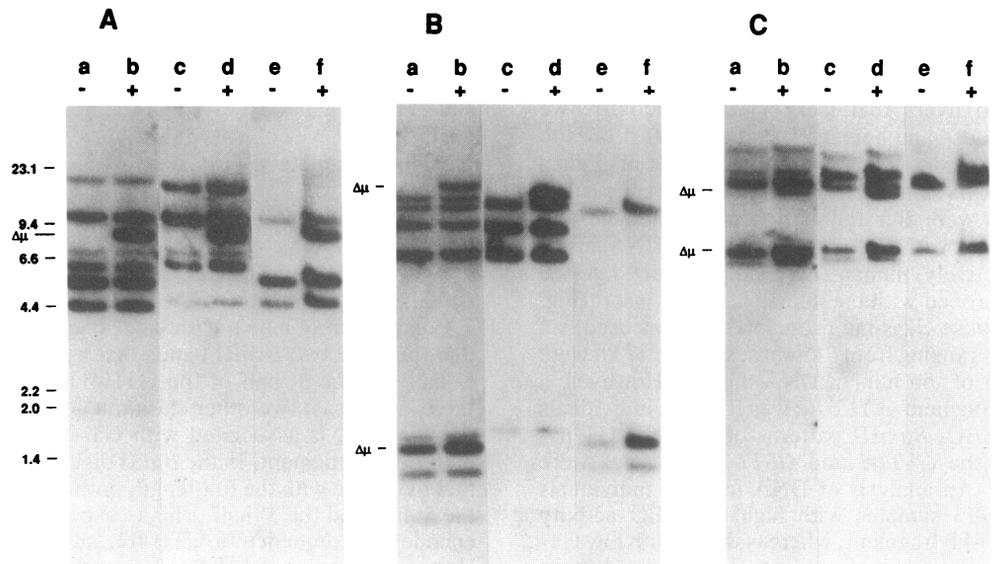

FIG. 4. Southern blot analysis of human class-mu GT genes with subfragments of GTH411. DNA samples from a low (0.1 nmol per min per ml of whole blood; − lanes, lanes a, c, and e) and a high (0.7 nmol per min per ml of whole blood; + lanes, lanes b, d, and f) GT-tSBO individual were digested with *Eco*RI (*A*), *Bgl* II (*B*), or *Bam*HI (*C*) and hybridized with portions of the GTH411 insert. Subclones containing the 5′ half and 3′ half of the cDNA insert were constructed by cutting the insert at a *Bgl* II site at nt 592. Lanes: a and b, hybridization with the full-length GTH411 insert (nt 1–1160); c and d, hybridization with the 5′ half of the GTH411 insert (nt 1–592); e and f, hybridization with the 3′ half of the GTH411 insert (nt 593–1160). The protein coding sequence in GTH411 extends from nt 19 to nt 669.

tSBO activity confirms the observation that the GT-tSBO enzyme is a member of the mu GT class. The data also show that the absence of GT-tSBO activity in an individual is due to an absence of a class-mu GT mRNA rather than the production of a mutant polypeptide. The absence of detectable class-mu mRNA in the livers of individuals with low GT-tSBO activity also suggests that, although there are several class-mu genes, there is only one class-mu GT mRNA expressed in human liver (the one that encodes GT-tSBO).

We also describe the isolation of a human class-mu GT cDNA clone that encodes the human GT isoenzyme GT-μ, expressed from the *GST1-1* genetic locus. This isoenzyme is closely related to the rat and mouse class-mu isoenzymes, but it is not possible to be certain which, if any, of the rodent enzymes are the homologues of human GT-μ. GT-μ is unusual because of its high specific activity on the substrate *trans*-stilbene oxide (5 μmol per min per mg of purified protein) (5). Purified rat GT 4-4 also has a high GT-tSBO activity (2 μmol per min per mg of protein), while the rat3-3 isoenzyme has an activity of 0.1 μmol per min per mg of purified protein (J.S., unpublished data).

Southern blot analysis of restriction digests of human DNA samples shows that individuals with GT-tSBO activity have additional *Bam*HI, *Bgl* II, and *Eco*RI fragments that hybridize with mouse and human class-mu GT cDNA clones. We have not observed changes in restriction fragment size that might indicate that the loss of GT-tSBO activity is due to a nucleotide substitution that creates or abolishes a restriction site. In addition to the 11.5-kb *Bam*HI fragment and the 8-kb *Eco*RI fragment that are associated with GT-tSBO activity, there are several other restriction fragments that hybridize with class-mu cDNA probes. The multiple bands seen in Figs. 3 and 4 are not due to cross-hybridization between the mouse class-mu cDNA probe and human class-alpha or class-pi GT genes; under the hybridization conditions that we have used, the mouse class-mu probe does not cross-hybridize with human class-alpha mRNA (Fig. 1) or with mouse or rat class-alpha cDNA clones (data not shown).

The data in Fig. 4 suggest that there are at least three class-mu GT genes in the genome of individuals expressing GT-tSBO, but there could be additional genes that we have not counted. If there are three genes, two that are found in every member of the population, and a third (*GST1*) that is found only in individuals expressing GT-tSBO, then we would expect a heterozygous individual with a single copy of the gene encoding GT-tSBO and two copies of the other two class-mu GT genes to have 25% more DNA that can hybridize with a class-mu GT cDNA probe. This value agrees with the 25–31% increase that we measured. Although there could be other class-mu GT genes, the mRNA and Southern blot hybridization data clearly show that the pGT875 and GTH411 probes hybridize with the class-mu gene that encodes GT-tSBO.

The observation that there are multiple class-mu GT genes in the human genome contrasts with earlier genetic studies that have identified a single *GST1* locus (8) and with the results reported here that individuals lacking GT-tSBO activity lack detectable liver class-mu GT mRNA. Our results suggest that there is a single gene that encodes both the liver isoenzyme GT-μ and the peripheral blood activity GT-tSBO and that this gene is deleted in about half of the population. This result is consistent with the observation that the GT-tSBO enzyme in peripheral blood is indistinguishable biochemically from liver GT-μ (5). We do not know whether the other *Eco*RI bands seen in Figs. 3 and 4 contain additional genes or pseudogenes, but if they encode other GT isoenzymes, the mRNA from these genes cannot be detected in human liver RNA samples from individuals lacking GT-tSBO. One of these bands could contain the *GST5* locus, which encodes a GT activity found in human brain (13). Multiple class-alpha and class-mu mRNAs have also been found in rat and mouse liver, often in response to inducers of detoxification enzymes such as phenobarbital (26) or dietary antioxidants (15, 16). The other human class-mu GT genes may be expressed in other tissues, or they may respond to specific inducers, as has been shown in the cytochrome P-450 isoenzyme family (28).

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