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Identification of Class-mu Glutathione Transferase Genes *GSTM1–GSTM5* on Human Chromosome 1p13

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Summary

The *GSTM1, GSTM2, GSTM3, GSTM4,* and *GSTM5* glutathione transferase genes have been mapped to human chromosome 1 by using locus-specific PCR primer pairs spanning exon 6, intron 6, and exon 7, as probes on DNA from human/hamster somatic cell hybrids. For *GSTM1,* the assignment was confirmed by Southern blot hybridization to a pair of 12.5/2.4-kb *Hin*dIII fragments. The *GSTM1*-specific primer pairs can be used to identify individuals carrying non-null *GSTM1* alleles. The organization of these five genes was confirmed by the isolation of a yeast artificial chromosome clone (GSTM-YAC2) that contains all five genes. With this clone, the location of the *GSTM1–GSTM5* gene cluster on chromosome 1 was confirmed by fluorescence in situ hybridization. Both regional assignment using the fractional length method and examination of probe signal with reference to R-banded chromosomes induced by BrdU places the gene cluster in or near the 1p13.3 region. The close physical proximity of the *GSTM1* and *GSTM2* loci, which share 99% nucleotide sequence identity over 460 nucleotides of 3′-untranslated mRNA, suggests that the *GSTM1*-null allele may result from unequal crossing-over.

Introduction

Chemical carcinogens, such as those derived from the benzo(a)pyrene in cigarette smoke, are formed and detoxified by a series of oxidation and conjugation reactions that can proceed through a maze of different pathways. Genetic differences that increase the level of the phase I cytochrome P450 enzymes or decrease the activity of the phase II glutathione transferase enzymes have been associated with increased risk of cancer (Ayesh et al. 1984; Seidegard et al. 1986, 1990; Sugimura et al. 1990; Strange et al. 1991; Hayashi et al. 1992). Although polymorphisms in detoxification genes can play an important role in determining cancer risk, relatively little is known about their genetics and expression. This is especially true for the human glutathione transferases.

The glutathione transferases (E.C.2.5.1.18) are a family of proteins that detoxify chemical carcinogens, either by binding them directly or by conjugating glutathione to reactive electrophilic sites (Jakoby 1978; Mannervik and Danielson 1988). The nonmicrosomal glutathione transferases have been grouped into four classes based on protein sequence similarity: alpha, mu, pi, and theta (Mannervik and Danielson 1988; Harris et al. 1991; Mannervik et al. 1992). Members of the same class share 75%–95% protein sequence identity. For example, most mammalian class-mu cDNA sequences share more than 85% sequence identity and can be used to identify many other class-mu mRNAs and genes under relatively stringent hybridization conditions. Members of different classes have 25%–30% protein sequence identity; class-mu glutathione transferase cDNA probes do not cross-react with mRNAs from other glutathione transferase classes.

Unlike the class-alpha glutathione transferases, which are found in all human livers, the major liver

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class-mu isoenzyme is found only in about half the adult population (Board 1981*a*, 1981*b*). This enzyme is encoded at the *GSTM1* locus, which displays three alleles: *GSTM1a*, *GSTM1b*, and *GSTM1*-null (Board 1981*a*, 1981*b*; Suzuki et al. 1987). Individuals who are homozygous *GSTM1*-null lack a glutathione transferase activity against the substrate *trans*-stilbene oxide in their peripheral blood (Seidegard and Pero 1985) and do not express either GSTM1a or GSTM1b in their livers (Seidegard et al. 1987; Widerstein et al. 1991; Mannervik et al. 1992). The *GSTM1*-null allele appears to be a deletion of the gene that encodes GSTM1a/b (Seidegard et al. 1988), since individuals who are homozygous *GSTM1*-null lack an 8-kb *Eco*RI fragment of genomic DNA that hybridizes with both the 5′ and 3′ half of a cDNA clone that encodes GSTM1b. The *GSTM1*-null deletion has been associated with lung cancer (Seidegard et al. 1986, 1990; Hayashi et al. 1992) and adenocarcinoma of the colon (Strange et al. 1991).

Glutathione transferase cDNAs from four additional class-mu loci have been described: *GSTM2* (Vorachek et al. 1991), *GSTM3* (Campbell et al. 1990), *GSTM4* (Zhong et al., in press), and *GSTM5* (Takahashi et al., in press). The cDNAs encoded by *GSTM1* and *GSTM2* share a remarkable 99% sequence identity (one substitution and two insertions) over 537 nucleotides (nt) of exon 8; 460 of these are 3′ noncoding (Vorachek et al. 1991). Thus, the demonstration that *GSTM1* and *GSTM2* are physically linked would suggest that the frequent deletion of the *GSTM1* locus is caused by unequal crossing-over.

Unambiguous mapping of the human class-mu glutathione transferase genes has been difficult, both because there are several closely related class-mu glutathione transferase loci and because at least one of these loci is polymorphic. In an initial report of the cloning of the *GSTM1* gene product, DeJong et al. (1988) presented in situ hybridization data suggesting that the *GSTM1* locus is found on chromosome 1p31. However, since in situ hybridization cannot distinguish between *GSTM1* and the four other class-mu loci, it is unclear whether *GSTM1* or some other class-mu glutathione transferase gene was responsible for the hybridization to chromosome 1 in the study by DeJong et al. (1988). While members of a multigene family can sometimes be mapped more accurately by Southern hybridization to DNA from human/rodent somatic cell hybrids, somatic cell mapping can be difficult when polymorphisms at the locus are very common, as is the case with *GSTM1*. Because *GSTM1* is missing from half the population, half of the human/rodent somatic cell hybrids that contain the appropriate chromosome lack the *GSTM1* gene.

To avoid problems in interpreting complex patterns of Southern blot hybridization, we developed locus-specific PCR primer pairs for the *GSTM1*, *GSTM2*, *GSTM3*, *GSTM4*, and *GSTM5* genes. With the primer pair specific for the *GSTM1* locus in a PCR reaction on genomic DNA, a signal was obtained only in those individuals or cell lines with a non-null allele at the *GSTM1* locus and only in human/hamster cell lines containing chromosome 1. Therefore, this *GSTM1*-specific primer pair can be used to determine whether an individual carries a non-null *GSTM1* locus. PCR amplification with primer pairs specific for the *GSTM2*, *GSTM3*, *GSTM4*, and *GSTM5* genes shows that these genes are located on chromosome 1 as well. In addition, we have isolated a yeast artificial chromosome (YAC) clone that contains all five of these loci, demonstrating that these genes are closely linked.

Material and Methods

Nomenclature

In this paper, we follow the new nomenclature recommended for human glutathione transferases (Mannervik et al. 1992). Thus, the locus of the polymorphic class-mu glutathione transferase expressed in liver, referred to as "*GST1*" in most earlier work (Suzuki et al. 1987; DeJong et al. 1988; Seidegard et al. 1988) is referred to as "*GSTM1*." The protein encoded by the *GSTM1a* allele and called "GT-μ" in earlier work is called "GSTM1a," and the product of the *GSTM1b* allele, previously called "GT-ψ," is now called "GSTM1b." The locus of the class-mu enzyme expressed in muscle, previously referred to as "*GST4*" (Suzuki et al. 1987; Vorachek et al. 1991), is now called "GSTM2"; a portion of this gene was also described as "*GSTmu3*" (Taylor et al. 1991). The class-mu gene expressed in brain (Suzuki et al. 1987; Campbell et al. 1990) is now referred to as "*GSTM3*." Recently, two additional cDNAs have been described, *GSTM4* (Zhong et al., in press), which was first isolated as *GSTmu2* (Taylor et al. 1991), and *GSTM5* (Takahashi et al., in press).

Reagents

^{32}P-dCTP was purchased from New England Nuclear or ICN Radiochemicals. Restriction enzymes were purchased from New England Biolabs, GIBCO/BRL, or Promega. DNA polymerase I and the Klenow fragment of DNA polymerase I were obtained from

Table 1

Oligonucleotides Used

Name[a]	Complement	Origin	Sequence	Reference
GSTM1E6	GSTM1E7	*GSTM1* exon 6	CCA AAG TAC TTG GAG GAA CTC CC	Seidegard et al. 1988
GSTM1E7	GSTM1E6	*GSTM1* exon 7	GAG ATG AAG TCC TTC AGA TTT	Seidegard et al. 1988
GSTM1I6	GSTM1E7	*GSTM1* intron 6	GCT TCA CGT GTT ATG GAG GTT C	Present report
GSTM2E6	GSTM1E7	*GSTM2* exon 6	CCA GAA TAC CTG CAG GCA CTC	Vorachek et al. 1991
GSTM2I6	GSTM2E6	*GSTM2* intron 6	GTA TGA CAA ATC TGT GGT GTC C	Present report
GSTM3E6	GSTM3E7	*GSTM3* exon 6	CCT CAG TAC TTG GAA GAG CT	Campbell et al. 1990
GSTM3E7	GSTM3E6	*GSTM3* exon 7	CAC ATG AAA GCC TTC AGG TT	Campbell et al. 1990
GSTM3I6	GSTM3E6	*GSTM3* intron 6	TAA GAT GCT TAG GTC TGA GG	Present report
GSTM4E4	GSTM4I5	*GSTM4* exon 4	GCA AGC ACA ACC TGT GTG AG	Taylor et al. 1991
GSTM4I5	GSTM4E4	*GSTM4* intron 5	GTA GAT GGG AAT ACA AGC CTG G[b]	Taylor et al. 1991
GSTM4I6	GSTM4E7	*GSTM4* intron 6	CCA TAC ATC CTA TGT TAC AGA GA	Present report
GSTM4E7	GSTM4I6	*GSTM4* exon 7	GGC TCA AAT ATA CGG TGG AG	Present report
GSTM5I6	GSTM5E7	*GSTM5* intron 6	TTA CTT CAT GTG TTT CGG GGT	Present report
GSTM5E7	GSTM5I6	*GSTM5* exon 7	GGC TCA AAT ATA CGC TTC AT	Present report

[a] Locus-specific primer pairs are underlined.

[b] This nucleotide was specified incorrectly when the primer was synthesized; it should be a C, to match the *GSTM4* gene.

Boehringer-Mannheim Biochemicals or Promega. PCR kits were purchased from Perkin Elmer Cetus. cDNA plasmid GTH411, the product of the *GSTM1* locus, has been described elsewhere (Seidegard et al. 1988).

Oligonucleotides

Oligonucleotide probes were prepared by Operon Technologies (San Pablo, CA) or by the University of Virginia Sequencing Center. The oligonucleotides used in these experiments are described in table 1.

DNA Samples

DNA from six individuals was used in many of the hybridization and PCR amplification experiments. Samples 4 and 9 come from individuals with low GT-tSBO activities who lack the 8-kb *Eco*RI fragment associated with the *GSTM1* locus; and samples 6 and 10 come from individuals with high GT-tSBO activity who have the 8-kb *Eco*RI fragment. Figures with lanes marked 4, 6, 9, or 10 display DNA from the respective individuals. DNA samples from two other individuals, simply denoted "+" and "−," were used for the lanes shown in the right-hand panel of figure 4 and in figures 6–8. A seventh DNA sample, which contained the 8-kb *Eco*RI fragment, was used for the PCR amplification that was subcloned to generate PCR-GSTM1, PCR-GSTM4, and PCR-GSTM5.

Somatic Cell Hybrids

Construction of somatic cell hybrids 9528c-1, 1881c-13b, and Raj 5 has been described elsewhere (Gardiner et al. 1988). The 10a series of hybrids (10a, 10a7a, 10a7b, and 10a7c) were obtained by fusion of Chinese hamster ovary nutritional mutant cells with human acute myelogenous leukemia (AML-M2) cells separated from erythrocytes and mature granulocytes on Ficoll hypaque gradients according to a method described elsewhere (Drabkin et al. 1985; Sacchi et al. 1986). Cytogenetic analysis was carried out using a two-part procedure consisting of trypsin-Giemsa banding followed by Giemsa-11 staining on the same metaphase spreads (Barton et al. 1991).

Nucleic Acids Methods

Purification of human DNA and RNA, hybridization and sequencing procedures, and measurement of GT-tSBO activity were as described elsewhere (Seidegard et al. 1988). Southern blots to nylon membranes were hybridized with random primer–labeled probe at 65°C in 0.5 M sodium phosphate buffer and were washed at 65°C in 40 mM sodium phosphate buffer according to the method of Church and Gilbert (1984).

PCRs were performed in a Perkin Elmer Cetus programmable temperature-cycling apparatus, using the Perkin-Elmer Gene-Amp reaction kit according to the manufacturer's recommendations. A standard cycle included denaturation at 95°C for 1 min, reannealing for 2 min at 55°C, and chain elongation with *Taq* polymerase at 72°C for 3 min. Most samples were amplified through 30–35 cycles. The exact number of cycles and primer concentrations for specific reactions is provided

in the figure legends. In addition to primers and template DNA, the amplification reaction contained 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM Mg^{++}, 200 μM of each dNTP, and 2.5 units of *Taq* polymerase. Some reactions used a "hot-start" procedure with the Perkin-Elmer Ampliwax product. In this procedure, the primers, Mg^{++}, and deoxynucleotides are separated from the DNA template and *Taq* polymerase by a paraffin plug until the reagents are heated to 75°C–80°C in the first cycle.

The PCR product that was used for subcloning clones PCR-GSTM1, PCR-GSTM4, and PCR-GSTM5 was obtained after 25 amplification cycles at 37°C. A portion of the mixture was then ligated into the plasmid pGEM7zf+ (Promega) that had been linearized at the *Sma*I site. The ligation mixture was then transformed into *Escherichia coli* strain 7118. The exon 6/intron 6/exon 7 fragment from *GSTM2* or *GSTM3* was subcloned into pGEM7zf+ after amplification with appropriate primers (table 1).

Isolation of YAC Clones

The YAC library from the laboratory of Maynard Olson (Burke et al. 1987; Brownstein et al. 1989) was screened by a PCR-based approach (Green and Olson 1990; Kwiatkowski et al. 1990) using primer sets GSTM1I6/GSTM1E7 (*GSTM1* specific) and GSTM-2E6/GSTM2I6 (*GSTM2* specific; table 1). Positive clones were colony purified and subjected to analysis as described in Results.

In Situ Hybridization

Fluorescence in situ hybridization (FISH) to human metaphase chromosomes was performed as described elsewhere (Patterson et al., in press). Concurrent banding of chromosomes analyzed by FISH was carried out by the method of Cherif et al. (1990). Fractional length measurements used the procedure of Lichter et al. (1990). Three separate FISH experiments were performed; one used BrdU banding as described by Cherif et al. (1990). At least 10 metaphase spreads were examined in each experiment. A positive FISH signal was scored if signals were seen on both chromatids of both chromosome homologues.

Results

Southern Blot Analysis of Human Class-mu GST Genes

In an earlier study (Seidegard et al. 1988), we examined the structure of the human class-mu glutathione transferase genes by using Southern blots of *Bam*HI, *Bgl*III, and *Eco*RI digestions of human DNA from more than 30 individuals. At that time we found only one polymorphism: the absence of one or more bands that correlated perfectly with the *GSTM1*-null allele. Since these three restriction enzymes did not discriminate adequately between human and hamster glutathione transferase genes in DNA from somatic cell hybrids, we used *Hin*dIII for these studies (fig. 1*A*). A total of eight *Hin*dIII bands are seen among individuals with a non-null *GSTM1* allele: H1/H2 (15.5 kb), H3 (12.5 kb), H4 (8 kb), H5 (7 kb), H6/H7 (5.5 kb), H8 (2.4 kb), and H9 (1.8 kb). Two of these bands, H3 (12.5 kb) and H8 (2.4 kb), are missing in individuals who are homozygous for the *GSTM1*-null allele. Thus, H3/H8 can be used to identify the *GSTM1* locus, as has been reported elsewhere (DeJong and Tu 1989). However, there is an additional pair of polymorphic bands (H4 and H5) that does not correlate with either GT-tSBO activity or the H3/H8 bands. The three possible combinations of H4 and H5 that are expected if these two bands represent two alleles at the same locus can all be seen in figure 1*A:* human DNA samples 4 ($GT\text{-}tSBO^-$) and 10 ($GT\text{-}tSBO^+$) each appear to be heterozygous with both H4 and H5, sample 9 (−) appears to be homozygous for H5, and sample 6 (+) appears to be homozygous for H4. This *Hin*dIII RFLP has been observed elsewhere (DeJong et al. 1991).

Despite the complexity of the pattern in figure 1*A*, it is possible to identify restriction fragments containing portions of human glutathione transferase genes in human/hamster somatic cell hybrids. Figure 1*B* and *C* shows the pattern of bands found when Chinese hamster DNA, human DNA, or somatic cell hybrids containing different sets of human chromosomes are probed with the *GSTM1b* cDNA. The H1/H2 pair is obscured by bands found in hamster DNA, but H3 and H8 can be seen in some hybrids. In addition to the H3/H8 pair, the H5, H6/H7, and H9 bands can be clearly distinguished from the hamster DNA background. Note that the H3/H8 pair may be missing either because a somatic cell hybrid lacks the human chromosome that contains the *GSTM1* locus or because the individual from whom the chromosomes were obtained has a deletion at the locus. For example, cell line 1881c contains the human H5 and H6/H7 bands (fig. 1*C*), which are associated with H3/H8 (fig. 1*B;* see below), but 1881c does not contain H3/H8; thus 1881c was derived from a *GSTM1*-null individual. In contrast, cell line 10a (fig. 1*C*) and cell line 10a7b (fig. 1*B*) contain H3/H8 and H6/H7, but the H5 band does not appear; presumably they are derived from an indi-

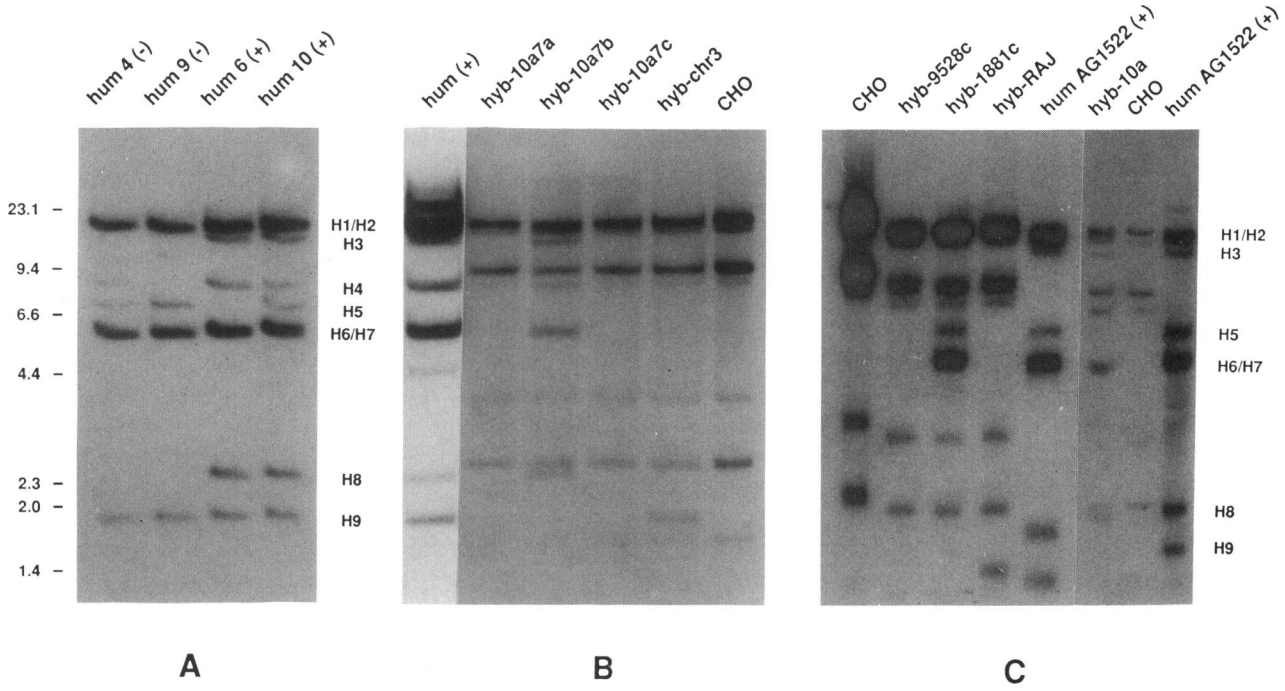

Figure 1 Southern blot analysis of class-mu glutathione transferase genes in human DNA and human/hamster somatic cell hybrids. All DNA samples were digested with *Hin*dIII, electrophoresed on an 0.8% agarose gel, transferred to a nylon membrane, and hybridized with the human GTH411 cDNA insert. *A*, DNA samples from individuals with low GT-tSBO activity (−; hum 4 and hum 9) and high GT-tSBO activity (+; hum 6 and hum 10). *B*, Somatic cell hybrid clones with and without chromosome 1. Lane 10a7a contains 5, 9p+, 13–15, 21, and 22; and lane 10a7c contains 5, 13–15, 18, 21, and 22. These are all sister clones from the same fusion and were derived by subcloning the hybrid designated hyb-10a. The 10a series of hybrids were derived from human cells from an AML patient carrying an 8;21 translocation. Also shown is the cell line UCTP-2a-3 (hyb-chr3), which contains an intact chromosome 3 as its only human material. *C*, Additional human, hamster, and somatic cell hybrid DNAs. The human chromosomes present in the somatic cell hybrids are as follows: 9528c—der 3 (3qter→3p24.2::21q21→21qter) from t(3;21), 6, 8–11, 13, 15, 20, 22, and X; 1881c—der 1 (1qter→1p31::21q21 →21qter) from t(1;21), 4, 8, 18, 19, 22, Y, and 1-2 small translocations (human;CHO); RAJ—3–5, t(7p;CHO), 10, 13, 16, fragment of 17q, 19, and der 21 (21pter→21q22::22q11→22qter; and 10a—1, 4–6, t(7p;CHO), der 8 (8pter→8q22::21q22→21qter), 9, 13–15, 18–21, der 21 (21pter→21q22::8q28qter), and 22. The derivations of these cell lines are described in detail in papers by Drabkin et al. (1985), Sacchi et al. (1986), and Gardiner et al. (1988). In panel *C*, the first lane ran more slowly than did lanes 2–5, so that the bottom two bands in lane 1 correspond to the bottom two lanes in lanes 2–4. Likewise, lane 5 ran more rapidly than did lanes 1–4, so that the bottom bands in lanes 4 (RAJ) and 5 (hum AG1522) both correspond to H9. Lanes 1–5 of panel *C* were run on a gel different from that in lanes 6–8.

vidual who carries the H4 band rather than the H5 band. Thus, the presence of the H3/H8 and the H5 bands is informative, but their absence is not.

All five of the independently isolated hybrids depicted in figure 1 show concordant presence or absence of chromosome 1 and the *GSTM1* locus. A total of 14 independent hybrids were analyzed similarly. Eleven showed concordance between *GSTM1* and chromosome 1. Of the three that were discordant, two had the chromosome but not the gene. These lines presumably carried a chromosome 1 with the *GSTM1*-null allele. Only one hybrid had the gene but lacked chromosome 1 by cytogenetic analysis, and this hybrid had several unidentifiable rearranged human chromosomes. No other human chromosome showed a higher concordance with the *GSTM1* locus.

We were unable to obtain from a non-null individual a somatic cell line that contained only chromosome 1, which would conclusively demonstrate that the *GSTM1* locus was found on chromosome 1. Instead, we examined three subclones of cell line 10a7: 10a7b, which contains chromosome 1 and other human chromosomes, and 10a7a and 10a7c, which do not contain chromosome 1 (fig. 1*B*). Subclone 10a7b, the only cell line containing human chromosome 1 in this panel, contains the 12.5-kb (H3) and 2.4-kb (H8) *Hin*dIII bands from the *GSTM1* locus (fig. 1*B*). 10a7b also contains the 5.5-kb H6/H7 bands from another class-mu

glutathione transferase locus. Thus, the *GSTM1* (H3/H8) locus and possibly several other class-mu glutathione transferase loci (H6/H7 and probably H4, which is obscured by a hamster band) are present on human chromosome 1. We note that hybrid cell lines 10a7a and 10a7c, both of which have lost the H3/H8 fragments that are diagnostic for the *GSTM1* locus, both retain chromosome 13 (DeJong et al. 1991). Since these hybrids clearly started out with the *GSTM1* locus but subsequently lost the locus while retaining chromosome 13, we conclude that chromosome 13 cannot be the location of *GSTM1*.

Cell line UCTP-2A-3, which contains only human chromosome 3, contains an additional class-mu glutathione transferase locus on a 1.8-kb *Hin*dIII fragment (H9; fig. 1*B*). The H9 band can also be seen in the RAJ hybrid, which contains chromosome 3 (fig. 1C), but not in the 9528c hybrid, which lacks only a small portion of chromosome 3 t(3;21), 3p24-3pter. The absence of the H9 band in 9528c suggests that H9 maps in the region 3p24-3pter.

Identification of Gene-specific PCR Amplification Primers

To simplify the assignment of the human *GSTM1* locus in human/hamster somatic cell hybrids, we identified locus-specific PCR primers by amplifying a portion of *GSTM1* containing exon 6, intron 6, and exon 7. From the structure of class-mu genes in rat (Lai et al. 1988) and mouse (Reinhart and Pearson, in press), we predicted that human intron 6 would be short (90 bp) and that exon 7 would contain the nucleotide substitution responsible for the difference between the *GSTM1a* and *GSTM1b* alleles. A 23-nt amplification primer from nt 388–410 (exon 6, primer GSTM1E6; table 1) and a 21-nt primer from residues 572–552 (exon 7, primer GSTM1E7) of the GTH411 insert were used to amplify human DNA (fig. 2). Three major bands are seen, at 380 bp, 275 bp, and 180 bp. The middle, 275-bp band is the exact size predicted if human intron 6 is 90 bp long and is in approximately the same location as it is in the rodent genes. The 180-bp band probably results from the amplification of a processed pseudogene lacking intron 6. The 380-bp band has not been characterized further. The DNA in figure 2 was amplified through 35 cycles at 55°C; when human DNA is amplified through 25 cycles, the 275-bp band is more abundant than the other two (data not shown). Since the pattern of amplification is similar in *GSTM1*-null and non-null individuals, the GSTM1E6/E7 primer pair must also be priming DNA amplification from loci other than *GSTM1*. The GSTM1E6/E7 primer pair also amplifies hamster DNA (data not shown).

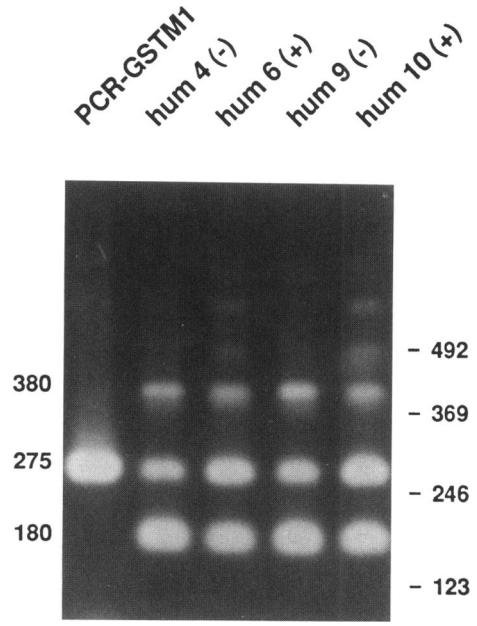

Figure 2 PCR amplification of human class-mu glutathione transferase genes. Sequences from nucleotides 388–410 (GSTM1E6) and 572–552 (complement and GSTM1E7) from the human *GSTM1b* cDNA clone GTH411 insert (Seidegard et al. 1988) were used to prime the PCR amplification of 2 μg of human genomic DNA from four individuals, two carrying null *GSTM1* alleles (lanes 4 and 9) and two carrying a non-null *GSTM1* allele (lanes 6 and 10) and 0.5 ng of the PCR-GSTM1 subclone. Primer concentrations were 1.6 μM. The DNA samples were amplified through 35 cycles with annealing at 55°C, and additional *Taq* polymerase was added after the 15th cycle.

The predicted structure of the 275-bp amplified band was confirmed by cloning and sequencing the products of a 25-cycle amplification of DNA from two individuals: one carrying a non-null *GSTM1* allele and one carrying the null allele (fig. 3). Each of the clones contains portions of exons 6 and 7 and an 87–90-nt intron 6. One of the sequences (PCR-GSTM1) is identical to the corresponding portion of the *GSTM1a* cDNA (DeJong et al. 1988). The two other sequences (PCR-GSTM4 and PCR-GSTM5) differ from the PCR-GSTM1 sequence both in the exon and intron sequences. PCR-GSTM5 matches the *GSTM5* (Takahashi et al., in press) cDNA sequence exactly; this sequence was also isolated from DNA sample 4, which is homozygous for the *GSTM1*-null allele. The PCR-GSTM4 sequence differs from the *GSTM4* sequence published by Zhong et al. (in press) at two nucleotides (fig. 3) that do not change the encoded protein.

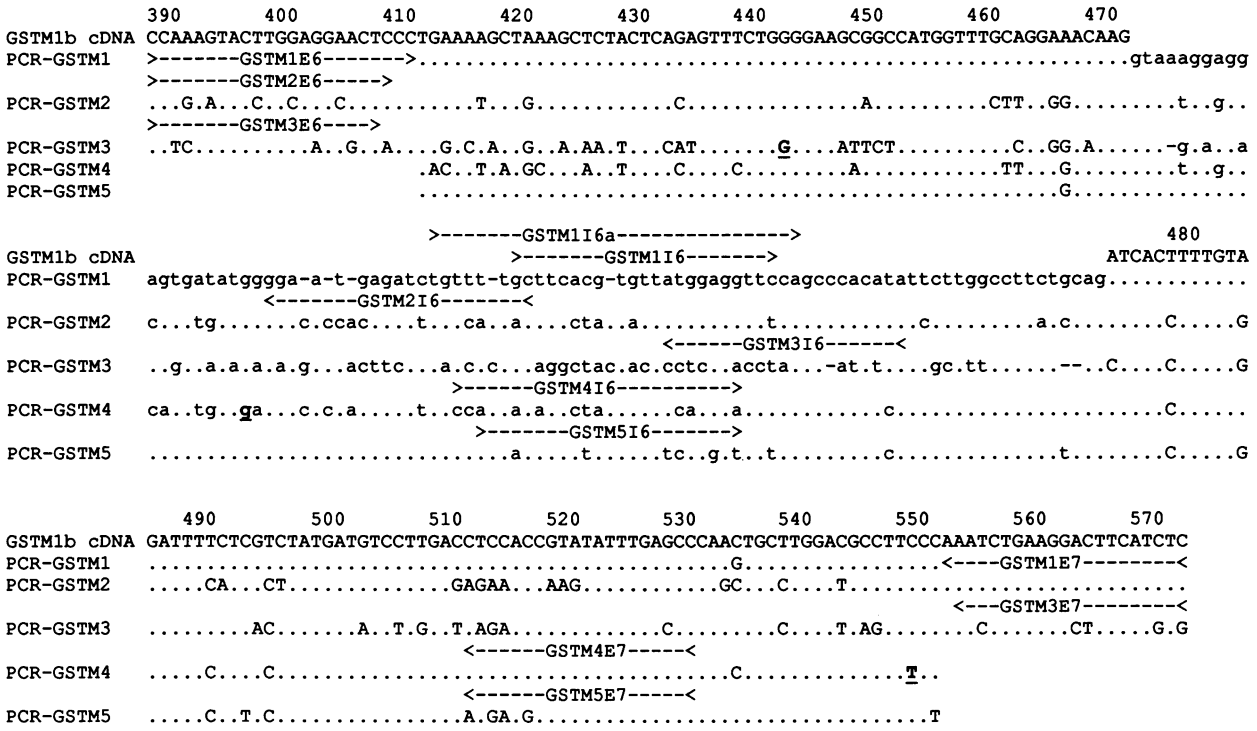

Figure 3 Nucleotide and amino acid sequences of class-mu glutathione transferase gene PCR products. Exon 6, intron 6, and exon 7 nucleotide sequences from *GSTM1-GSTM5* genes are shown. Differences between the GSTM1b cDNA sequence (Seidegard et al. 1988) and the five sequences are indicated; identities are denoted by periods. Intron 6 sequences are shown in lowercase letters. There are no differences between the *GSTM2* cDNA sequence (Vorachek et al. 1991) and PCR-GSTM2, which was produced by the GSTM2E6/GSTM1E7 primer pair, or between the *GSTM5* cDNA (Takahashi et al., in press) and PCR-GSTM5, which was produced by GSTM1E6/E7. One difference between the GSTM3 cDNA sequence (Campbell et al. 1990) and the PCR-GSTM3 sequence (GSTM3E6/GSTM3E7 primer pair) and two differences between *GSTM4* (Zhong et al., in press) and PCR-GSTM4 are underlined. The positions of the locus-specific amplification primers are shown.

The intron 6 sequences shown in figure 3 allowed us to construct primer pairs (GSTM1I6a/E7 and GSTM1I6/E7; table 1) that amplify only DNA containing a non-null *GSTM1* locus (fig. 4). GSTM1I6a (fig. 3) differs from the PCR-GSTM5 sequence at seven positions and from the PCR-GSTM4 sequence at 11 positions; GSTM1I6 differs from the PCR-GSTM5 and PCR-GSTM4 sequences at 6 and 11 positions, respectively. When GSTM1I6a or GSTM1I6 is used with the GSTM1E7 exon primer, a 157- or 151-nt fragment is amplified only in individuals carrying a non-null *GSTM1* allele (fig. 4). (The gel in the left-hand panel of fig. 4 is overloaded, so that some nonspecific amplification is seen from individuals who lack a non-null *GSTM1* allele.)

The GSTM1I6/E7 primer-pair is *GSTM1* specific: it does not amplify *GSTM1*-null human DNA (fig. 4) or Chinese hamster DNA (figs. 4, *right* and 5); it does not amplify the paralogous regions of *GSTM2* and *GSTM3* in the PCR-GSTM2 and PCR-GSTM3 clones (fig. 4, *right*) or in the PCR-GSTM5 and PCR-GSTM4 clones (data not shown); and it does not amplify the class-mu glutathione transferase gene on chromosome 3. These PCR experiments confirm the Southern blotting results (fig. 1); when chromosome 1 is lost from a cell line containing the *GSTM1* locus, the *GSTM1* locus is lost as well.

Other Class-mu Glutathione Transferase Genes Are Found on Chromosome 1

We also constructed primers from exons 6 and 7 of *GSTM2* and *GSTM3* to determine the intron 6 sequences from these loci (table 1 and fig. 3). For *GSTM2*, the sequence of exon 6 and exon 7 matched exactly the cDNA sequence that we had determined (Vorachek et al. 1991). For *GSTM3*, there was a single amino acid discrepancy at residue 146 in the protein sequence; a T→G replacement changes the Trp at residue 146 in the published sequence (Campbell et al.

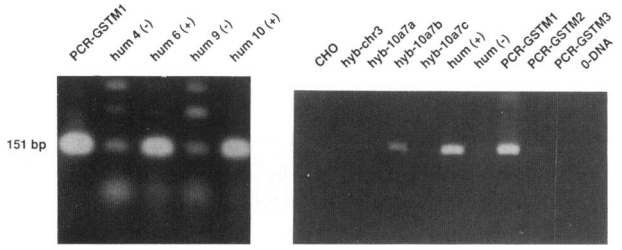

Figure 4 Detection of the *GSTM1* locus by using PCR amplification. *Left panel,* PCR amplification of DNA from individuals expressing low (hum 4 and hum 9) and high (hum 6 and hum 10) GT-tSBO activities by using the GSTM1I6a/E7 primer pair. Amplification from 0.5 ng of clone PCR-GSTM1 DNA is also shown. DNA samples were amplified as in fig. 2. The sizes of the amplified fragments are shown. *Right panel,* Detection of the *GSTM1* locus in human DNA and somatic cell hybrids by using the GSTM1I6/E7 primer-pair. One hundred nanograms of human DNA, 200 ng of somatic cell hybrid DNA, and approximately 10 pg of plasmid DNA were used. These amplifications were performed for 35 cycles with renaturation at 55°C; primer concentrations were 1.0 μM.

1990) to a Gly. Gly is found at this position in the other published human class-mu glutathione transferase sequences.

Determination of these five intron 6 sequences allowed us to construct oligonucleotides that detect each of the different glutathione transferase loci (table 1 and fig. 3). Figure 5 shows the results of amplifications using the locus-specific primer pairs listed in table 1. We cannot demonstrate directly that the *GSTM2, GSTM3, GSTM4,* and *GSTM5* primer pairs are locus specific, because none of these loci is deleted in the human population. However, several lines of evidence support their specificity. (1) Each of these primer pairs differs from the other genes that we have sequenced, in at least 25% of the sites in one of the two primers; the *GSTM1*-, *GSTM2*-, and *GSTM3*-specific primer pairs differ at more than 40% of the positions in the intron primer. (2) Except for *GSTM4* and *GSTM5,* the locus-specific primer pairs do not amplify plasmid DNA containing any of the other PCR subclones. The *GSTM4* and *GSTM5* primers do cross-amplify to a limited extent with the other PCR subclones; however, the *GSTM4*-specific primer pair amplifies the PCR-GSTM4 plasmid, the cell line 10a7b, and the human DNA samples much more efficiently than it does the heterologous plasmids. In addition, the pattern of amplification with a second *GSTM4*-specific primer pair (GSTM4E4/I5; table 1), matches the GSTM4I6/E7 pattern exactly. (3) None of the primer pairs amplifies the class-mu glutathione transferase gene on chromosome 3. (4) YAC clones containing different subsets of class-mu glutathione transferase genes amplify only with subsets of the primer pairs (see below). Thus, human chromosome 1 contains the *GSTM1, GSTM2, GSTM3, GSTM4,* and *GSTM5* loci.

Both the *GSTM2* and *GSTM3* primer pairs produce two bands on the human DNA samples. One of the *GSTM2* bands is the same size as that seen when the PCR-GSTM2 subclone is amplified; this band is found in the 10a7b somatic cell hybrid (fig. 5) and in the GST-YAC2 and GST-YAC3 (fig. 6) YAC clones. The other GSTM2 band is smaller and slightly lower in intensity; this band appears not to be present on chromosome 1. This pair of bands is not seen in figure 5; we attribute the difference to the use of a hot-start protocol for PCR amplification in figure 5; a conventional protocol was used in figure 6. Likewise, the *GSTM3* primer pair

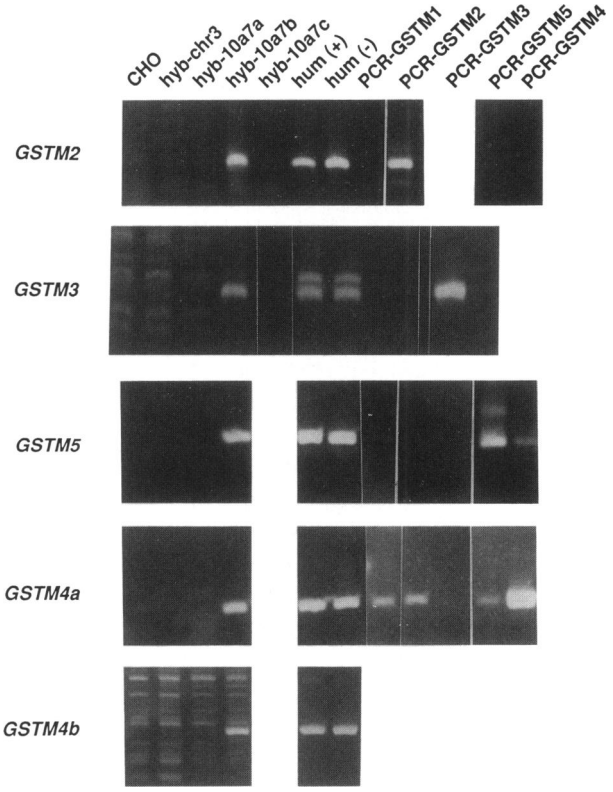

Figure 5 Detection of *GSTM2, GSTM3, GSTM4,* and *GSTM5* in somatic cell hybrids. The samples described in the right-hand panel of fig. 4 were amplified with the locus-specific primer pairs summarized in table 1. GSTM4a was amplified with the GSTM4I6/E7 primer pair; and GSTM4b used GSTM4E4/I5. Amplifications were performed for 35 cycles with renaturation at 55°C, by using 1.0 μM primers, 100 ng of human DNA, 200 ng of hamster or somatic cell hybrid DNA, and 10 pg of plasmid DNA.

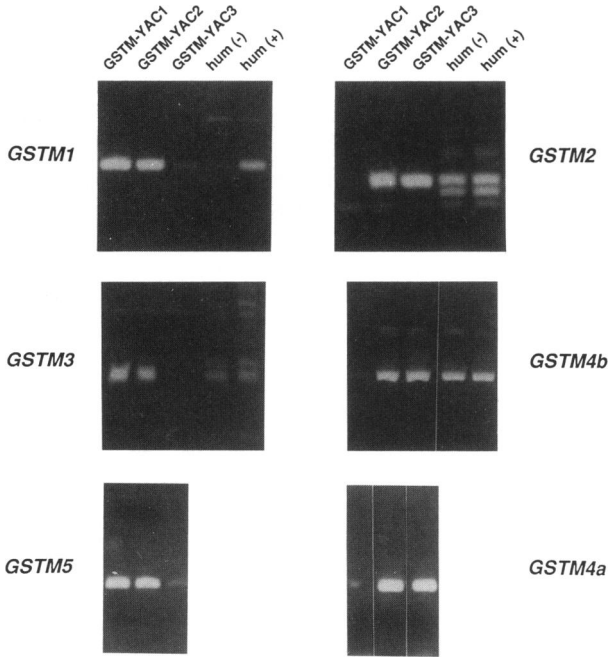

Figure 6 Detection of *GSTM1, GSTM2, GSTM3, GSTM4,* and *GSTM5* in GSTM-YAC clones. The locus-specific primer pairs shown in table 1 were used to amplify DNA samples isolated from the three YAC clones, through 35 cycles with renaturation at 55°C and with 1.0 μM primers. GSTM4a was amplified with the GSTM4I6/E7 primer pair; and GSTM4b used GSTM4E4/I5. Amplification was performed as described in the legend to fig. 5.

produces two bands; here the smaller band is found on chromosome 1, while the larger is elsewhere. Additional class-mu glutathione transferases are expressed in muscle (Singh et al. 1988) and brain (GST6, Suzuki et al. 1987). These additional bands, while not from chromosome 1, may correspond to these other class-mu loci. Although the UCTP-2A-3 cell line contains a class-mu glutathione transferase gene (or pseudogene) on human chromosome 3, this gene is not one of the *GSTM1–GSTM5* loci.

Isolation of YAC Clones Containing Class-mu Glutathione Transferase Loci

The GSTM1I6/E7 (*GSTM1*-specific) and GSTM-2E6/I6 (*GSTM2*-specific) primer pairs were used to screen a human YAC library (Burke et al. 1987). Roughly 60,000 clones were screened with the two primer pairs; three independent isolates were colony purified. GSTM-YAC1, -YAC2, and -YAC3 contain 370-, 530-, and 300-kb artificial chromosomes, respectively. These clones were characterized both by Southern blot hybridization (fig. 7) and by PCR amplification (fig. 6).

Southern blotting shows that GSTM-YAC2 contains the 8-kb *Eco*RI fragment and the 12.5/2.4-kb (H3/H8) *Hin*dIII fragments associated with the *GSTM1* locus, as well as H1/H2, H5, and H6/H7. Each of the bands in the *Eco*RI digest of YAC DNA comigrates with human *Eco*RI bands—as is expected, since the human YAC4 library was made from an *Eco*RI partial digest. However, there is an 8-kb *Hin*dIII band in GSTM-YAC1 and a 5-kb *Hin*dIII fragment in GSTM-YAC3 that may contain junctions with the YAC vector (the latter band, below H6/H7, is not apparent in the reproduction but is clearly separated on the original film). Southern blot hybridization (not shown) suggests that the H4/H5 polymorphism corresponds to the PCR-GSTM5 locus. GSTM-YAC1 also contains the 8-kb *Eco*RI fragment and the 2.5-kb *Hin*dIII fragment, but it appears to lack the 12.5-kb *Hin*dIII fragment associated with the *GSTM1* locus. GSTM-YAC3 lacks the 8-kb *Eco*RI fragment and the 12.5/2.4-kb *Hin*dIII fragments of *GSTM1*, but it contains the H1/H2 and H6/H7 fragments, as well as an additional *Hin*dIII band.

PCR analysis of the YAC clones shows that GSTM-YAC2 contains all of the class-mu glutathione transferase genes for which we have locus-specific primer pairs:

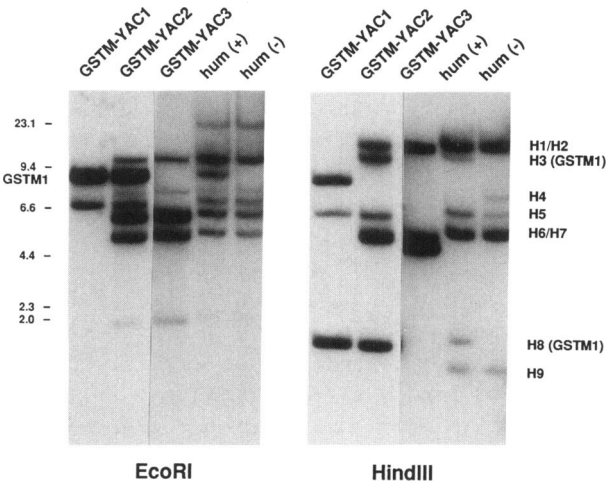

Figure 7 YAC clones containing human class-mu glutathione transferase genes. Southern transfer hybridizations of *Eco*RI and *Hin*dIII digests of DNA from three YAC clones are shown. Digested DNA samples were separated on a 0.8% agarose gel. Fifteen micrograms of human DNA and approximately 0.5 μg of yeast miniprep DNA were loaded on each lane. The Southern transfer was probed with the GTH411 insert (*GSTM1*).

GSTM1, GSTM2, GSTM3, GSTM4, and *GSTM5;* this result is consistent with the Southern blot shown in figure 7. Likewise, GSTM-YAC1 contains *GSTM1, GSTM3,* and *GSTM5,* while GSTM-YAC3 contains *GSTM2, GSTM3,* and *GSTM4.* The isolation of these three YAC clones confirms that these five class-mu glutathione transferase genes are physically linked.

To confirm the mapping of the GSTM gene cluster to chromosome 1, we used the GSTM-YAC2 clone, which contains the entire *GSTM1–GSTM5* class-mu gene cluster, to probe metaphase spreads of human chromosomes by using FISH. Three separate FISH experiments (more than 30 metaphases) were carried out; in one experiment (more than 10 metaphases), R bands were induced by BrdU and stained with DAPI. A positive FISH signal was scored only if it was seen on both chromatids on both chromosome homologues; by this criterion, only chromosome 1 was positive. Background labeling was negligible.

The band position of the probe was determined by two methods. (1) Four cytogeneticists evaluated independently the preparations and placed the signal at 1p13.3-21 by visual examination of the signal with reference to R-banded chromosomes induced by BrdU and stained by DAPI. (2) With unbanded chromosome preparations, the fractional length position of the probe signal (FLpter; fig. 8) was calculated by measuring projected images of 10 chromosomes, as described by Lichter et al. (1990). The FLpter range was 0.43–0.47, with an average value of 0.45, placing the signal at 1p13.1-1p13.3. Taken together, these data confirm the somatic cell mapping; when all the data are considered, the most likely location for the GSTM gene cluster is at 1p13.3. These assignments differ from the earlier mapping by DeJong et al. (1988), which placed the band at 1p31, several bands (and therefore at least 10 million bp) away from 1p13.3. The earlier assignment is based on a relatively low-resolution in situ hybridization study using a radioactively labeled *GSTM1* cDNA probe. Our FISH results have considerably higher resolution; the gene clearly maps nearer to the centromere. The 1p13.3 assignment was confirmed recently by linkage analysis (NIH/CEPH Collaborative Mapping Group 1992; Shan et al. 1992).

Discussion

The human class-mu glutathione transferase loci *GSTM1–GSTM5* have been mapped to chromosome 1. Our results are consistent with earlier reports of Southern blot hybridizations to somatic cell hybrids (Islam et al. 1989; DeJong et al. 1991; Shan et al. 1992). We have also identified a *Hin*dIII restriction-fragment polymorphism at a second class-mu glutathione transferase locus, which may correspond to the *GSTM5* locus. The isolation of the GSTM-YAC2 clone, which contains the *GSTM1–GSTM5* loci, offers the strongest evidence that all five genes are linked on chromosome 1. However, since the literature on class-mu glutathione transferase mapping is confusing, it is worthwhile to rank the certainty of our conclusions while comparing our data with those obtained by other investigators.

The GSTM1 *Locus Is Found on Chromosome 1*

This conclusion is the strongest. It is supported by Southern blot hybridization to somatic cell hybrids, by PCR amplification with locus-specific hybrids, and by FISH to human metaphase chromosomes. The mapping to chromosome 1 is also consistent with earlier mapping results (DeJong et al. 1988, 1991), although not with the conclusions drawn by DeJong et al. (1991). The incorrect assignment of *GSTM1* to chromosome 13 is based on a 15% discordance for an assignment to chromosome 1, which reflects the fact that two cell lines that contained chromosome 1 lacked the 8-kb *Eco*RI fragment associated with *GSTM1.* The assignment of *GSTM1* to chromosome 13 reflected the fact that four cell lines displaying the 8-kb band contained chromosome 13 and that no cell lines that contained chromosome 13 lacked the 8-kb band. However, since a cell line may lack the 8-kb band because it is derived from an individual lacking the 8-kb band, the lacks-the-band/has-the-chromosome discordance is not informative. If this type of discordance is removed from DeJong et al.'s analysis, the 8-kb *Eco*RI band associated with the *GSTM1* locus can be assigned to chromosomes 1, 3, 6, 13, 15, 17, 18, or 19, with no discordance. Our results from cell lines 10a7a and 10a7c (figs. 1*B* and 4) show clearly that *GSTM1* is not found in cell lines containing chromosome 13 but lacking chromosome 1. The assignment of *GSTM1* to chromosome 1 is also supported by a large linkage study on chromosome 1 (NIH/CEPH Collaborative Mapping Group 1992) and by another somatic cell mapping study (Shan et al. 1992). We are not aware of any data that are inconsistent with the assignment of *GSTM1* to chromosome 1.

The GSTM2, GSTM3, *and* GSTM4 *Loci Are Also Found on Chromosome 1*

Taylor et al. (1991) have isolated a cosmid containing *GSTM2* and *GSTM4.* Our PCR-amplification data

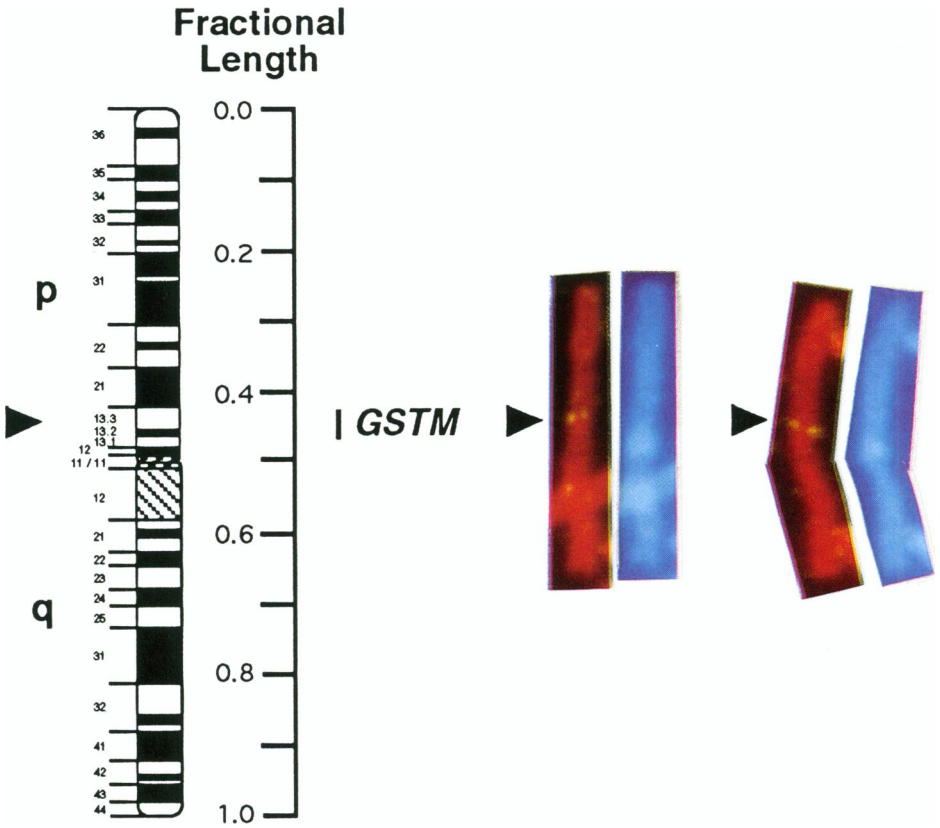

Figure 8 FISH with GSTM-YAC2: idiogram of chromosome 1 with a graph showing FLpter map location of the GSTM gene cluster. The position of the FITC (*yellow*) GSTM-YAC2 hybridization signal on the propidium iodide (*red*) chromosome was measured from the pter end and was divided by the measured length of the chromosome. The pter end was assigned by DAPI (*blue*) staining of the same chromosome; DAPI preferentially stains the q arm. The arrowheads indicate the position of the GSTM gene cluster as detected by FISH. The chromosomes shown are the two chromosomes 1 from the same metaphase spread. In each chromosome pair the left-hand (*red*) image depicts the propidium iodide staining of the chromosome when analyzed by FISH. The right-hand (*blue*) image shows unbanded counterstaining of the same chromosome with DAPI.

show that *GSTM1* is near *GSTM2* and *GSTM4*. *GSTM4* has been mapped to chromosome 1 by using a different set of locus-specific primers (Zhong et al., in press). The PCR data show also that *GSTM3* is present with *GSTM1* in this cluster. *GSTM3* encodes an unusual class-mu glutathione transferase that is relatively distantly related to the other class-mu genes (less than 75% nucleotide sequence identity); thus, primers for *GSTM3* are unlikely to cross-react with the other class-mu loci. The *GSTM3*-specific primer pair does not amplify from plasmid DNAs containing the paralogous sequences of *GSTM1*, *GSTM2*, *GSTM4*, or *GSTM5*, and it does not amplify from the UCTP-2A-3 cell line, which contains human chromosome 3. Thus, we are confident that the GSTM3E6/I6 primer pair is locus specific and that *GSTM3* is near *GSTM1*, *GSTM2*, and *GSTM4* on chromosome 1.

A Class-mu Glutathione Transferase Gene Is Found on Chromosome 3 in the Region 3p24-3pter

Mannervik has reported that a class-mu glutathione transferase gene or pseudogene is present on chromosome 3 (Islam et al. 1989). The human H9 (1.8-kb *Hin*dIII) band is found in the UCTP-2A-3 cell line, which contains chromosome 3 as its only human DNA, but not in line 10a7, which contains chromosome 1. The t(3;31) translocation in cell line 9528c apparently resulted in the loss of the H9 band, which suggests that H9—and therefore this class-mu gene—maps in the region 3p24-3pter.

The GSTM5 Locus Is Found on Chromosome 1

This conclusion is based on the specificity of the GSTM5I6/E7 primer pair. This primer pair does not amplify from the other PCR subclones or from GSTM-

YAC3. In addition, PCR-GSTM5 hybridizes preferentially to the polymorphic H4/H5 pair of *Hin*dIII bands (data not shown); H4 can be seen in GSTM-YAC1 and GSTM-YAC2 but not in GSTM-YAC3. Because of an endogenous 8-kb *Hin*dIII band in hamster DNA, we cannot confirm that the 10a7b cell line contains H4; however, cell line 1881c, which also contains H6/H7, clearly contains H5, the other allele at this locus. Thus, both the specificity of the GSTM5I6/E7 primers and the correlation of H4/H5 with H3/H8 and H6/H7 suggest that *GSTM5* is found on chromosome 1.

Five different human class-mu glutathione transferase genes, *GSMT1–GSTM5,* are adjacent on chromosome 1. In our earlier report on the *GSTM1* polymorphism (Seidegard et al. 1988), we suggested that there were at least three class-mu glutathione transferase genes; this is clearly an underestimate. In addition to the five class-mu genes on chromosome 1, a gene or pseudogene is present on chromosome 3, and other genes may be found on chromosome 6 (DeJong et al. 1991). The pairs of bands seen in human DNA with the *GSTM2* and *GSTM3* primer pairs suggest additional genes. Several class-mu isoenzymes have been characterized from muscle (Singh et al. 1988; Hussey et al. 1991), aorta, and heart (Tsuchida et al. 1990), and it is likely that additional class-mu enzymes are expressed in other tissues. There may be as many as four class-mu glutathione transferase genes that have not yet been identified.

The proximity of *GSTM1* and *GSTM2,* which share 99% nucleotide sequence identity, suggests that the high frequency of the *GSTM1*-null allele (about 0.7; Board et al. 1990) may be the result of unequal crossing-over. There are now many examples of deletions caused by unequal crossing-over, including deletion of the CYP21B gene (resulting in adrenal hyperplasia; Sinnott et al. 1990), deletion of β-globin gene family members (resulting in thalassemias; Metzenberg et al. 1991), deletion of growth hormone genes (Vnencak-Jones and Phillips 1990), and formation of hybrid proline-rich protein genes. In these cases, a region of 400 or more nucleotides with greater than 98% identity was implicated in the unequal recombination event. *GSTM1* and *GSTM2* are 99% identical over at least 537 nucleotides; this identity may extend into intron 7 or beyond the poly-A addition site. Recent data in our laboratory suggest that *GSTM1* is located on the 3′ side of *GSTM2;* thus, if the two genes were to recombine within exon 8, the 5′ half of *GSTM1* would be deleted in the hybrid *GSTM2/1* gene. The hybrid glutathione transferase formed would be indistinguishable from the *GSTM2* gene product because in exon 8 there are no amino acid differences between *GSTM1* and *GSTM2.* Recombination in the 3′ exon could explain the asymmetry of the crossing-over event; a *GSTM2* deletion has never been reported. A similar *5′/3′* hybrid and deletion has been seen with the proline-rich protein genes. We are exploring the possibility that the deletion of *GSTM1* because of unequal crossing-over has occurred several times in humans. Nevertheless, to date we have seen only two polymorphic loci: *GSTM1,* with its three alleles, and the apparent H4/H5 alleles at *GSTM5.* If the class-mu genes on chromosome 1 are closely linked, then the *GSTM1*-null deletion(s) must be relatively small; we have never seen any evidence for other deleted class-mu glutathione transferase genes.

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