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Expression of Tandem Glutathione S-Transferase Recombinant Genes in COS Cells for Analysis of Efficiency of Protein Expression and Associated Drug Resistance

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SUMMARY

Expression vectors were designed and constructed to achieve optimum production of two different isozymes of rat glutathione S-transferase (GST) (EC 2.5.1.18) in COS cells, for studies of drug resistance. Promoter-enhancer elements from the simian virus 40 (SV40) early-region or the mouse α_2(I)-collagen gene, GST cDNAs encoding the rat Ya or Yb_1 isozymes, and an SV40 replicative origin (*ori*) were positioned in the vector to express two GSTs at high levels in the same cell. The optimized construct yielded levels of both GST proteins (1% of postmitochondrial protein fraction) that were up to 1.3-fold greater than the sum of those produced individually by two single-unit expression constructs. The best production of the tandem recombinant gene products was observed when the genes were placed in a head to head orientation in close proximity (1 kilobase). With the recombinant genes configured in this way, the plasmid DNA was also amplified in COS cells to higher levels (30% increase over single-unit expression constructs), as *ori* elements were placed on both DNA strands. Cells expressing the recombinant GSTs were viably sorted by flow cytometry on the basis of a GST-catalyzed conjugation of glutathione to monochlorobimane. Sorted COS cells that expressed both GST Ya and Yb_1 from recombinant genes in a tandem, head to head configuration were 25 or 70% more resistant to the alkylating agent chlorambucil than cells that expressed GST Ya or Yb_1 alone.

The GSTs are a family of isozymes that can catalyze the covalent addition of the intracellular tripeptide glutathione to a structurally diverse array of physiological and xenobiotic electrophiles (1–3). Mammalian cytosolic GSTs are composed of two subunits (23–28 kDa each), which dimerize by noncovalent interactions and which display kinetic independence (4, 5). In rats, at least eight different monomeric subunits have been described, which in several cases are the products of different genes from several gene families (6). These monomers dimerize to yield homodimeric or heterodimeric isozymes, with substrate specificities that can be widely overlapping or specific and exclusionary (7).

Previously, our work (8, 9) and the work of others (10, 11) showed that expression of an exogenous GST isozyme in mammalian cells can confer resistance to structurally diverse alkylating molecules. The degree of resistance is related to the substrate specificity of an isozyme, as determined by kinetic analyses *in vitro* (9, 11). These results provide an explanation for the resistance to alkylating agents that is observed in human tumors (12, 13). Furthermore, expression of exogenous GSTs has also provided a means to study GST-mediated protection against DNA alkylation in cell and animal studies of toxicity and tumorigenesis (8, 14, 15).

Metabolism of several structurally dissimilar substrates by enzymes expressed from two recombinant genes in the same cell would be useful for studies of GST (8–11) or cytochrome P450 (16–18). This approach would also be particularly well suited for studies of drug metabolism using transgenic mice (14), because of the expense and lead time required to generate the animals. In a broader application, coexpression of two cDNAs in the same cell could be used in metabolic studies that require sequential reactions involving two enzymes, either to activate a cellular toxin of interest, such as a diol-epoxide (cytochrome P450 and epoxide hydrolase); to deactivate a cell toxin, such as methylglyoxal (glyoxalase I and II); or to reconstruct a multistep biosynthetic pathway, such as steroidogenesis (19).

Our goal here was to construct and analyze for expression efficiency, in COS monkey cells, plasmids that encode tandem

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ABBREVIATIONS: GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter; SV40, simian virus 40; *ori*, SV40 replicative origin; kb, kilobases.

configurations of two GST cDNAs, those for Ya and Yb_1. We show that positioning of the SV40 promoter-enhancer and *ori* in the expression vector is important for efficient GST expression. The biological impact of increasing intracellular GST activity on resistance to alkylating drugs was also examined.

Materials and Methods

Cell culture. COS-M6 monkey kidney cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Cytotoxicity assays were performed as previously described (9).

Construction of expression vectors. The pGTB38 (20) and pGTA/C44 (21) plasmids (Fig. 1) were gifts of Dr. Cecil Pickett, Merck Frosst Centre, Montreal. Construct 1 was prepared as previously described (8), in which a 342-base pair *Hind*III-*Pvu*II fragment from SV40, containing the viral replicative origin and early-region promoter-enhancer, was inserted immediately 5′ to the GST Ya ATG codon. The SV40 late-region polyadenylation signal was inserted immediately 3′ to the GST Ya cDNA in construct 1. Construct 2 was prepared by replacing the Ya cDNA in construct 1 with a 708-base pair *Hae*II-*Bam*HI fragment from construct Ob, which contained the GST Yb_1 cDNA. For construct 3, a 2.0-kb *Hind*III fragment from pAZ1003 (22), which contained the promoter-enhancer elements from the mouse α_2(I) collagen gene, replaced the SV40 early-region fragment of construct 2. For the remaining constructs in Fig. 1, single recombinant genes were arranged in the indicated tandem arrangements, using available restriction sites.

Electroporation. COS cells were electroporated with supercoiled plasmids, as described (9). Cells (2×10^6) were mixed with 10 μg of plasmid in 0.5 ml of phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) containing 5% fetal bovine serum. The electroporated cells were seeded into 100-mm dishes, and the medium was changed 24 hr later. Approximately 25% of the cells survived electroporation.

Fluorescence-activated cell sorting. Two days after electroporation, COS cells were trypsinized, rinsed, and suspended in phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) that was supplemented with 10% fetal bovine serum to decrease clumping during the sorting. Recombinant GST^+ COS cells (20% of the population) were separated (sorted) from negative control cells by a FACStar^{PLUS} (Becton Dickinson) that was equipped with argon and krypton lasers. The concentration and time of exposure of cells to the fluorescent labeling reagent monochlorobimane were optimized to ensure peak separation of GST^+ and GST^- cells. To judge the stringency of the sorting technique, COS cells were electroporated with construct 1 and sorted. Cells recovered from both the GST Ya^- and GST Ya^+ sort windows were immunostained with an antibody specific for the encoded rat GST Ya. Less than 1% of the cells in the GST Ya^- window and greater than 96% of the cells in the GST Ya^+ window were detectably immunostained. Details of the sorting technique are presented elsewhere (23a).

RNA and protein analyses. Forty-eight hours after electroporation, cells were trypsinized, and total cellular RNA, used for dot blot analysis, was isolated by density gradient centrifugation with guanidinium isothiocyanate-cesium chloride (23). Aliquots from the same RNA dilutions were applied to three nitrocellulose filters, which were then hybridized with a GST Ya, GST Yb_1, or human β-actin probe. For Western blot analysis of GSTs, postmitochondrial supernatants (16,500 $\times$ *g*, 20 min) of sonicated cells were electrophoresed and immunoblotted as described (8).

Results

GST Ya and Yb_1 expression in single and tandem configurations. GST was expressed from cDNAs encoded in plasmids that are replicated to high copy numbers when introduced into COS cells. Binding of constitutively expressed T antigen to the SV40 *ori* mediates the amplification (24, 25). When GST expression plasmids were introduced into COS cells

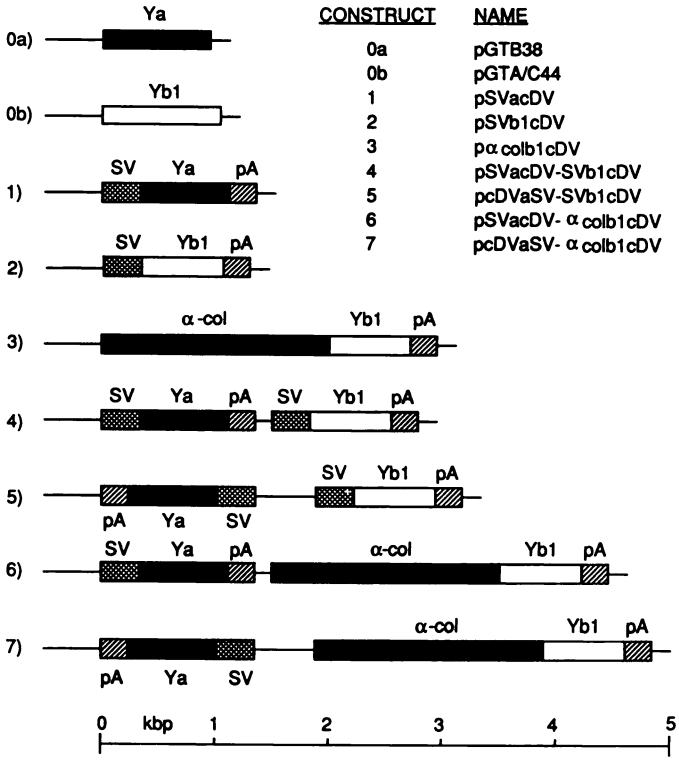

Fig. 1. Schematic maps of expression vectors containing single or tandem recombinant genes. *Thin lines*, pBR322 sequences; *boxed elements* are indicated (see Materials and Methods). *SV*, SV40 early-region promoter-enhancer fragment containing the viral replicative origin; *Ya* and *Yb_1*, full-length cDNA sequences encoding the respective GST subunits; *pA*, polyadenylation signal from SV40 late region; *α-col*, fragment containing promoter-enhancer from a mouse α_2(I)-collagen gene. In constructs 5 and 7, tandem recombinant genes are arranged in an inverted configuration on opposite DNA strands.

by electroporation, about 20% of the cells surviving 48 hr later actually expressed immunodetectable levels of cytosolic GST (8). The degree of GST expression was quantified by analysis of total cellular RNA and postmitochondrial supernatant protein that were prepared from groups of surviving COS cells 48 hr after electroporation with each of the plasmids shown in Fig. 1.

Northern blot analysis of RNA isolated from electroporated COS cells indicated that Ya and Yb_1 RNA species of the predicted size, 1.1 kb, had been synthesized (data not shown). No endogenous RNA of COS cells was detectable when either the Ya or Yb_1 probe was used to screen the blot (Fig. 2, constructs Oa and Ob). Single recombinant genes containing the SV40 early-region promoter-enhancer and the Ya or Yb_1 cDNA produced greatly abundant RNA species (Fig. 2, constructs 1 and 2). No detectable cross-hybridization between alternate probes and RNA samples was observed. No detectable level of Yb_1 RNA was produced in COS cells, a monkey kidney cell line, 48 hr after electroporation of an unamplifiable plasmid (Fig. 2, construct 3) that contained the mouse α_2(I)-collagen promoter and Yb_1 cDNA but that lacked the SV40 *ori*. Levels of RNA expressed from the α_2(I)-collagen-promoted Yb_1 recombinant gene were influenced by the position and orientation of the SV40 promoter-enhancer element. When the SV40 element was moved into closer proximity (by 1 kb) to the α_2(I)-collagen promoter, with the inverted orientation (from its position in construct 6 to that in construct 7, Fig. 1), levels of Yb_1-specific

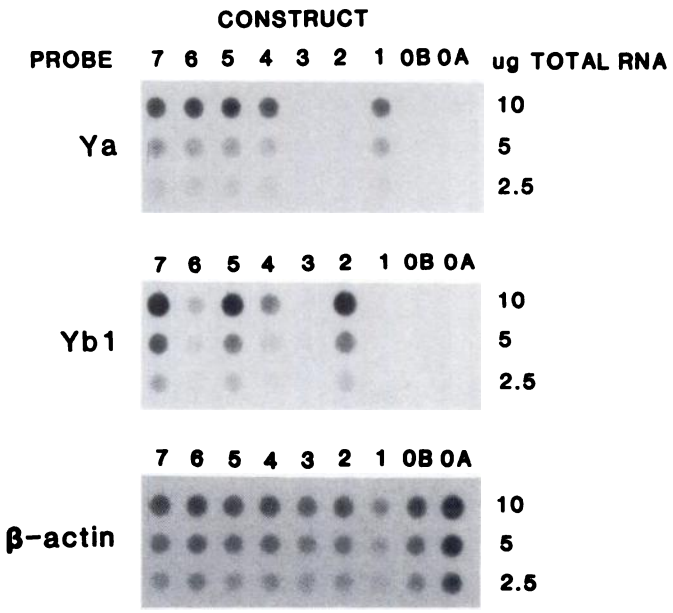

Fig. 2. Dot blot analysis of GST-specific RNA isolated from COS cells 48 hr after electroporation with the indicated GST expression vector (*CONSTRUCT*; see Fig. 1 for key). Electroporation and RNA isolation were done as described in Materials and Methods. The indicated amounts of total cellular RNA were applied to three replicate filters, which were then hybridized with the indicated probes. In the dishes harvested for RNA assays, the percentage of COS cells observed to be expressing recombinant GST 48 hr after electroporation was determined to be 20% by immunostaining analyses, done as described (8).

RNA increased 4.6-fold (from 0.14 to 0.64 units; Fig. 2, construct 6 versus construct 7, and Fig. 3). A similar dependence of expression efficiency upon enhancer position and orientation was observed for an SV40-promoted Yb_1 recombinant gene (Fig. 1, constructs 4 and 5). Levels of Yb_1-specific RNA were increased 2.7-fold (from 0.31 to 0.84 units; Fig. 2, construct 4 versus construct 5) when the distance between the two SV40 elements was reduced by half, to 1 kb, with the SV40 element in close proximity and in the inverted orientation.

Data generated by Western blot analysis corroborated results obtained by analysis of RNA. Integration of densitometric areas from GST Ya and Yb_1 protein standard curves (Fig. 4B) indicated that levels of protein expression derived from the SV40-promoted Yb_1 or Ya cDNAs were 1–3 ng of GST/μg of post-mitochondrial supernatant protein (Fig. 4A, constructs 1 and 2). Thus, one recombinant GST represents about 0.5% of the cytosolic protein fraction of COS cells. Expression levels of GST Yb_1 from construct 3, which contained the α_2(I)-collagen promoter without SV40 enhancer or *ori* sequences, were below immunodetectable limits (Fig. 4, construct 3). In agreement with the analysis of RNA, when an SV40 promoter-enhancer element was positioned in close proximity (in the inverted orientation) to either the α_2(I)-collagen-promoted Yb_1 cDNA (Fig. 1, construct 7 relative to construct 6) or the SV40-promoted Yb_1 cDNA (Fig. 1, construct 5 relative to construct 4), levels of expressed GST Yb_1 protein increased 2- or 11-fold (Fig. 3, 0.04 units to 0.09 units or 0.10 units to 1.11 units), respectively. As levels of GST Ya and Yb_1 protein expression from construct 5 indicate, COS cells do not appear to be limited in their capacity to produce both Ya and Yb_1 concomitantly at levels 1.3-fold greater than those observed for the expression of Ya or Yb_1 alone (Fig. 4, construct 5 versus constructs 1 or 2).

Southern blot analysis (Fig. 5) of plasmid DNA isolated from COS cells 48 hr after electroporation with constructs 0a–7 showed that SV40 *ori*-containing plasmids were replicated to high copy numbers (14,000–20,000 copies/average cell in electroporated populations in which 20% of the cells are known to express recombinant GST by immunostaining analysis) of *Dpn*I-resistant (26) plasmids (Fig. 3). Resistance of the plasmids to *Dpn*I digestion indicated that the plasmid copies were generated by replication in COS cells of groups 1, 2, and 4–7 and were not merely carried along as a contaminant within the dish for the 48-hr period after electroporation, as was the case for groups 0a, 0b, and 3 (Fig. 5). The principal finding that emerges from the analysis of plasmid copy number is that plasmids that contain two SV40 *ori* elements on the same DNA strand (Fig. 3, construct 4) are replicated to a copy number that is no greater than the levels of replicated plasmid that contain only one *ori* element (Fig. 3, construct 1). If, however, the *ori* elements are on opposing DNA strands (Fig. 3, construct 5), then the copy number is increased 1.10–1.30-fold (Fig. 3, construct 5 versus construct 4 and construct 7 versus construct 6).

Analysis and sorting of recombinant GST$^+$ COS cells by FACS. Based on observed levels of recombinant GST DNA, RNA, and protein expression, we estimated that GST activity present in COS cells expressing both GST Ya and Yb_1 would be about 2-fold greater than that in cells expressing only one GST subunit. Knowing that the degree of cellular resistance conferred to alkylating drugs is a function of cellular GST activity levels (9), we hypothesized that cells expressing both GST Ya and Yb_1 would be about 2-fold more resistant to the drug chlorambucil than cells expressing only GST Ya or only GST Yb_1. To quantify GST activity levels present in electroporated cells expressing single or tandem recombinant genes, we used a technique recently developed in our laboratory (9) that not only enables us to measure GST activity on a single-cell basis but also enables us to sort the recombinant GST$^+$ cells from electroporated populations containing both GST$^+$ and GST$^-$ cells. Two days after electroporation of construct 2 or construct 5, a FACS was used to viably sort the 20% of the COS population that expressed recombinant GST Yb_1, or Ya and Yb_1 (Fig. 6, B or C). Monochlorobimane, a hydrophobic fluorescent labeling reagent and substrate of GSTs (27), was used to enable the FACS to identify recombinant GST-expressing COS cells. The GSTs present in COS cells catalyzed the conjugation of glutathione to monochlorobimane, generating a highly fluorescent product. The cellular fluorescence intensity was a function of cellular GST activity (data not shown). Because conditions affecting the concentration of monochlorobimane (1 μM) and length of time cells were exposed to monochlorobimane (1 min) were unchanged during cell sorting and analysis, the cellular GST activity or number of GST molecules/cell determined the degree to which $Ya^+Yb_1^+$ cells fluoresced which was 5 to 10 times more intensely than the Yb_1^+ cells (Fig. 6, C versus B). When the length of time that GST $Ya^+Yb_1^+$ cells were exposed to monochlorobimane was increased from 1 min (Fig. 6C) to 2 min (Fig. 6D), the GST $Ya^+Yb_1^+$ cells appeared to become more well resolved. Presumably, there was a range of GST activity levels in GST $Ya^+Yb_{11}^+$ cells, and some cells required an additional minute to catalyze conjugate formation until some factor (e.g., glutathione content) became limiting. Different levels of GST activity in COS

CONSTRUCT	RELATIVE GST RNA LEVEL[a] Ya	RELATIVE GST RNA LEVEL[a] Yb1	RELATIVE GST PROTEIN LEVEL[b] Ya	RELATIVE GST PROTEIN LEVEL[b] Yb1	PLASMID COPY #[c] COPIES/CELL	CHLORAMBUCIL CYTOTOXICITY[d] LD_{90} (μg/ml)	CHLORAMBUCIL CYTOTOXICITY[d] RELATIVE RESISTANCE
0a) Ya	0.02	ND[e]	<.02	ND	<700	16.9	1.0
1) SV Ya pA	1.00 (11.50)[i,j] ±1.10	ND	1.00 (15.84)[f,k] ±0.92	ND	14,000	23.7	1.4
2) SV Yb1 pA	ND	1.00 (12.90)[i,k] ±3.56	<.02	1.00 (5.40)[f,k] ±0.50	----[g]	21.8	1.3
3) α-col Yb1 pA	ND	ND	<.02	0.06	<700	----[h]	----
4) SV Ya pA SV Yb1 pA	1.18	0.31	0.78	0.10	15,700	----	----
5) SV Yb1 pA / pA Ya SV	1.28 (14.70)[i,j] ±1.00	0.84 (10.80)[i,k] ±2.60	1.13 (17.90)[f,k] ±0.96	1.11 (5.99)[f,k] ±0.51	20,400	24.8	1.5
6) SV Ya pA α-col Yb1 pA	1.13	0.14	0.86	0.04	15,600	----	----
7) α-col Yb1 pA / pA Ya SV	1.12	0.64	0.97	0.09	17,100	----	----

FOOTNOTES

[a] Relative levels of Ya RNA and Yb_1 RNA were derived by densitometry of RNA dot blots shown in Fig. 2. Values were normalized relative to the β-actin hybridization in each sample. Values represent the average of three observations from three separate experiments. Because of differences in probe specific activity, no comparison of levels of Ya versus Yb1 mRNA can be made.

[b] Relative levels of Ya protein and Yb_1 protein were derived by densitometry of appropriate bands on western blots shown in Fig.4. Values represent the average of three observations from three experiments.

[c] Values are derived by densitometry of appropriate bands on Southern blot shown in Fig. 5.

[d] Relative resistance is expressed as the LD_{90} of the transfectants (constructs 1,2,5) divided by the LD_{90} of the control (construct 0a). Colony-forming assays were done in triplicate. Statistical significance was determined by analyses of covariance; curves for constructs 1,2 and 5 were significantly different from their control curves ($P<0.001$).

[e] Not detectable.

[f] Units = ng GST/100μg postmitochondrial supernate protein; mean and standard deviation.

[g] Not measured.

[h] Not tested.

[i] Units = arbitrary densitometer units; mean and standard deviation.

[j] $P < 0.05$

[k] $P > 0.05$

Fig. 3. Summary of the expression efficiency of single or tandem recombinant GST genes and associated resistance to chlorambucil.

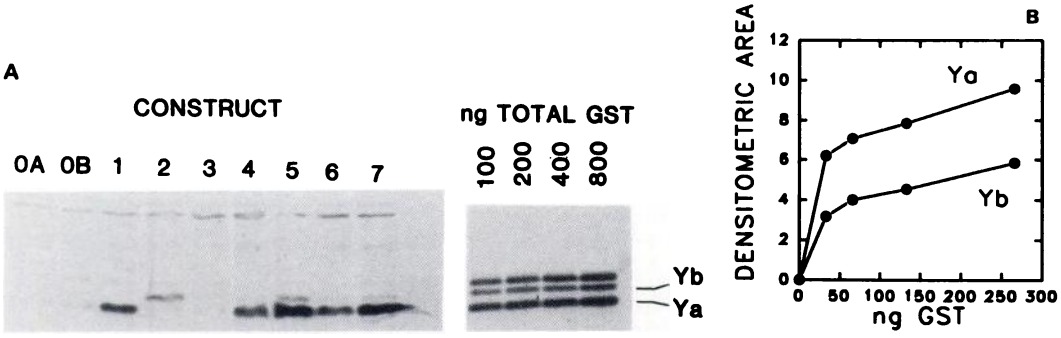

Fig. 4. Western blot analysis of GST Ya and Yb_1 protein levels in COS cells 48 hr after electroporation with the indicated GST expression vector (*CONSTRUCT*; see Fig. 1). Electroporation and preparation of postmitochondrial supernatants were done as described in Materials and Methods. A, Fifty micrograms of supernatant protein from samples 0a–7 were added to the indicated wells. Known amounts of a pool of rat liver GSTs were used as standards; Ya and Yb protein bands are indicated. B, The respective band densities are plotted. Silver staining of a replicate gel showed equal staining intensity for Ya and Yb bands. As in Fig. 2, cells expressing a recombinant GST represented 20% of the total cells in a dish. GST Ya and Yb expression was detected with polyclonal antiserum raised in rabbits against purified rat liver GST.

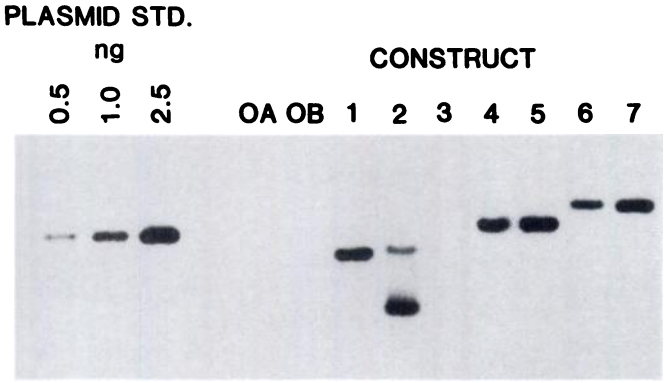

Fig. 5. Southern blot analysis of plasmid copy number in COS cells 48 hr after electroporation with constructs 0a–7. Known amounts of construct 0a were loaded in the *three left lanes* to act as copy number standards. Cellular DNA was extracted from electroporated COS cells 48 hr after electroporation. Cellular and plasmid DNA samples were treated sequentially with 100 μg of RNase A, 60 units of *Eco*RI, and 10 units of *Dpn*I. One microgram of DNA was then loaded in *lanes 0a–7*. The blot was probed with a 688-base pair *Bgl*I-*Ssp*I fragment from pBR322. Intensity of DNA bands was measured by densitometry, and the copy number was determined by comparing the intensity of sample bands with that of standard bands. For construct 2, incomplete *Eco*RI digestion resulted in both linear and supercoiled bands.

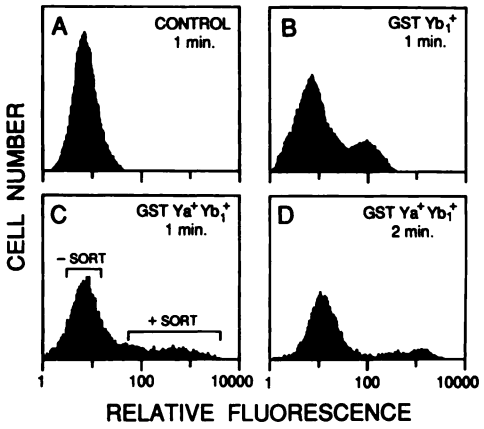

Fig. 6. FACS histograms of COS cells expressing recombinant GSTs. Cells were electroporated with constructs 0a (A), 2 (B), or 5 (C and D). Cells were harvested 48 hr later and then analyzed and sorted on a FACStar^{PLUS} after on-line exposure to 1 μM monochlorobimane for 1 min (A, B, and C) or 2 min (D). – *SORT*, gate from which the recombinant GST$^-$ cells were collected. + *SORT*, gate from which the recombinant GST$^+$ cells were collected.

cells can be explained by different levels of GST protein expression, which is consistent with interclonal variation in SV40 T antigen expression in COS cells (24). To sort GST Ya$^+$Yb$_1^+$ and GST Ya$^-$ Yb$_1^-$ cells from an electroporated population containing both types of cells, the population was exposed to 1 μM monochlorobimane on-line for 1 min, and fluorescence-channel gates were established (Fig. 6C) to collect purified cells for use in colony-forming assays. Purity of the sorted populations was verified by immunostaining. Assays of total glutathione content (data not shown) demonstrated that sorted populations, both the negative control and the GST$^+$ cells, had equivalent glutathione contents the day after the sort, that is, the day of the cytotoxicity assay.

Drug resistance conferred by single or tandem expression of GSTs. Thinking that the effect upon drug resistance of addition of more GST molecules/COS cell would be additive,

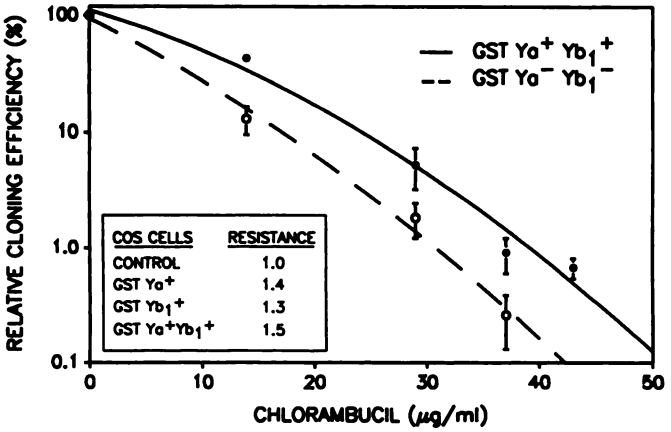

Fig. 7. Survival of sorted GST$^+$ COS cells after exposure to chlorambucil. Cells were electroporated with construct 0a, 1, 2, or 5, sorted 48 hr later, and then seeded. The day after seeding, sorted cells were exposed to chlorambucil for 1 hr in a colony-forming assay done in triplicate. Colonies were stained at 11 days and scored. The sorted GST Ya$^+$Yb$_1^+$ population had a 1.5-fold increase in resistance to chlorambucil, relative to the GST Ya$^-$ Yb$_1^-$ control. *Inset*, values of relative resistance to chlorambucil were measured in assays of sorted populations that expressed Ya, Yb$_1$, or Ya and Yb$_1$. Resistance values were derived from sets of curves that are significantly different from each other ($p < 0.001$). The control population (GST Ya$^-$Yb$_1^-$) consisted of either (i) cells that were electroporated with construct 0a, (ii) cells that were electroporated with construct 1 in which a frameshift was introduced into the Ya cDNA, thus disabling it, or (iii) GST$^-$ cells from flow sorting.

we hypothesized that the increase in cellular resistance due to the tandem expression of Ya and Yb$_1$ would equal the sum of resistances observed for expression of Ya or Yb$_1$ alone. Sorted populations of GST-expressing COS cells were exposed to a representative alkylating drug, the nitrogen mustard chlorambucil, in colony-forming assays to determine the degree of resistance conferred by recombinant GSTs. The colony-forming assays were done in triplicate, and the degree of protection observed in the Ya$^+$, Yb$_1^+$, or Ya$^+$Yb$_1^+$ cells was measured as an increase in resistance, relative to recombinant GST$^-$ COS cells, at an LD$_{90}$ drug concentration. The LD$_{90}$ is the concentration of drug that is required to kill 90% (10% survival) of the cells in a population, as measured by a colony-forming assay. When sorted GST Ya$^+$ or Yb$_1^+$ and recombinant GST$^-$ COS cells were exposed to chlorambucil for 1 hr in colony-forming assays, the Ya$^+$- or Yb$_1^+$-expressing populations had a 1.4-fold or 1.3-fold increase in resistance to chlorambucil, respectively, relative to controls (Fig. 7, *inset*). When sorted cells expressing both the Ya and Yb$_1$ cDNAs (Fig. 1, construct 5) were exposed to chlorambucil in a colony-forming assay, the GST Ya$^+$Yb$_1^+$ cells were 1.5-fold more resistant than the GST Ya$^-$Yb$_1^-$ cells (Fig. 7). The relative resistance value of 1.5, although greater than that for GST Ya$^+$ cells (1.4) or GST Yb$_1^+$ cells (1.3), was less than the sum of the Ya$^+$- and Yb$_1^+$-conferred resistance values (1.7).

Discussion

Our goal here was to identify an expression vector design that would enable the optimum production of two different GST isozymes in COS cells for studies of drug metabolism and resistance. By manipulation of promoter-enhancer/cDNA cassettes, we assembled a plasmid for tandem expression that yielded levels of both GST proteins that were as great as those produced by two single-unit expression constructs. The most

efficient production of both tandem gene products was seen when they were placed in a head to head orientation in close proximity (1 kb); this configuration also resulted in amplification of plasmid DNA to higher levels, as *ori* elements were placed on both plasmid DNA strands. Cells expressing the recombinant GSTs were viably sorted by flow cytometry on the basis of a GST-catalyzed conjugation of glutathione to monochlorobimane. Finally, we demonstrated that sorted COS cells that expressed GST Ya and Yb_1 from recombinant genes in a tandem inverted configuration were 25 or 70% more resistant, respectively, to the alkylating agent chlorambucil than cells that expressed GST Ya or Yb_1 alone.

Expression of functional GSTs was required for analysis of the capacity of GSTs to confer resistance to alkylating drugs. A sorting technique was developed, which separates the recombinant GST^+ COS cells from the GST^- cells, based on cellular enzyme activity levels of Ya, Yb_1, or Ya and Yb_1. Clearly, expression of tandem recombinant GST genes produced functional GSTs, as observed in the 5–10-fold increased capacity to catalyze the covalent addition of glutathione to monochlorobimane in sorted COS cells (Fig. 6C). Data from other laboratories indicate that cytosolic GSTs must be dimeric to be catalytically active (2), but exactly what kinds of dimers form among the recombinant Ya and Yb_1 subunits, and endogenous COS cell subunits are not known. Once this point is addressed, it is conceivable that tandem expression of two different GST subunits with nonoverlapping or minimally overlapping substrate specificities could be designed to confer cellular resistance to an array of structurally diverse toxins.

As a preliminary step toward characterizing the kinds of GST dimers formed and their stoichiometries of formation, as well as characterizing the specificities of GST isozymes for detoxification of toxins, we simply expressed single or tandem recombinant genes in COS cells to determine whether tandem expression of GSTs would be more efficient in conferring drug resistance than expression of single GSTs. We do not have an explanation as to why a doubling of recombinant GST protein content (to 1% of postmitochondrial supernatant protein) from expression of tandem GST Ya and Yb_1 recombinant genes provided a 1.5-fold increase in resistance to chlorambucil, a value that is 30% less than the sum of individually conferred resistance values for Ya (1.4) and Yb_1 (1.3). The simplest explanation for this result is that some factor in the drug detoxification pathway (e.g., glutathione) became limiting during the cytotoxicity assays. Another possible explanation is that a GST $YaYb_1$ dimer was preferentially formed in cells that expressed tandem genes and that the degree of specificity of $YaYb_1$ for inactivation of chlorambucil was less than the specificities of dimers present in cells expressing one recombinant gene (e.g., YaYa, Yb_1Yb_1, or a hybrid rat/monkey heterodimer). More work is required to determine which of the recombinant GST species in these cells are conferring the drug resistance.

COS cells can clearly support the combined biosynthesis of both the Ya and Yb_1 proteins at a level at which they together represent about 1% of the postmitochondrial protein (Fig. 4). In silver-stained gels of GST-expressing and control COS postmitochondrial fractions (data not shown), the Ya and Yb_1 bands were just distinguishable, and no diminution in intensity of any other COS band was detected. Relatedly, this lack of impact upon the synthesis of normal cell proteins was also observed in an *Escherichia coli* host (28), when GST π was produced at a level representing 20–40% of the soluble protein.

The results presented here showing tandem expression of GST support the broader goal of using GST overexpression as a means to trap and detoxify electrophilic molecules. GST-conferred protection has potential applications either in diminishing alkylation-induced carcinogenesis (28) or perhaps in producing normal, human, hematopoietic stem cells with increased resistance to alkylating antineoplastic drugs like chlorambucil (29), thus increasing the efficacy of the drug regimen by diminishing host cell toxicity. The tandem production of two distinct GST isoforms could then provide broader resistance to multiple, structurally diverse substrates, or it could simply provide greater resistance to one molecule such as chlorambucil (Fig. 7). Future studies with cell and transgenic animal models of GST expression will help to determine the role that GST-catalyzed conjugation can play in modulating electrophile-induced toxicity in mammalian cells.

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