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Expression of Human Microsomal Epoxide Hydrolase in *Saccharomyces cerevisiae* Reveals a Functional Role in Aflatoxin B_1 Detoxification

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The metabolism and genotoxicity of the carcinogenic mycotoxin, aflatoxin B_1 (AFB), was studied in the lower eukaryotic yeast *Saccharomyces cerevisiae*. Recombinant strains of yeast were engineered to express human cDNAs for CYP1A1, CYP1A2, and microsomal epoxide hydrolase (mEH). Coexpression of mEH with CYP1A1 or CYP1A2 resulted in significant decreases in measurements of AFB genotoxicity. In cells expressing CYP1A2 and mEH, the level of AFB-DNA adducts was decreased by 50% relative to cells expressing CYP1A2 alone. Mitotic recombination, as assayed by gene conversion at the *trp5* locus, was diminished by 50% or greater in cells coexpressing mEH and CYP1A2 compared to CYP1A2 alone. The mutagenicity of AFB in the Ames assay was also decreased by approximately 50% when AFB was incubated with microsomes containing CYP1A1 or CYP1A2 and mEH versus CYP1A1 or CYP1A2 alone. The biotransformation of AFB by CYPs is known to involve the generation of a reactive epoxide intermediate, AFB-8,9-epoxide, but previous direct biochemical and kinetic studies have failed to demonstrate any functional role for mEH in AFB detoxification. By reconstructing a metabolic pathway in intact yeast, we have shown, for the first time, that mEH may play a role in mitigating the carcinogenic effects of AFB.

Key Words: **aflatoxin B_1; microsomal epoxide hydrolase; cytochrome P450; genotoxicity; *Saccharomyces cerevisiae*.**

The aflatoxins are a structurally related group of mycotoxins that are found in grains and cereals stored under conditions favoring growth of fungal *Aspergillus spp.* (reviewed by Eaton and Heinonen, 1997). The most potent compound is aflatoxin B_1 (AFB), a known hepatocarcinogen in numerous animal species. Epidemiological evidence implicates AFB in development of hepatocellular carcinoma (HCC) in humans (Eaton and Heinonen, 1997). Aflatoxin B_1 must be biotransformed by cytochrome P450 (CYP) enzymes to the reactive intermediate AFB-8,9-epoxide (AFBO) to exert its carcinogenic effect. There are two stereroisomers of AFBO (*endo-* and *exo-*), and it is *exo*-AFBO that is capable of reacting with DNA, preferentially producing N^7-guanine adducts. Significant species differences exist in susceptibility to AFB-induced carcinogenesis. For example, rats are highly sensitive, whereas mice are quite resistant. The resistance of mice to AFB hepatocarcinogenicity appears to be due solely to the constitutive expression of a specific form of glutathione *S*-transferase (GST), mGSTA3-3, with unusually high catalytic activity toward AFBO (Buetler *et al.*, 1992). While conjugation to glutathione by GSTs is one pathway for AFBO elimination, another potential mechanism is hydrolysis of AFBO to the corresponding dihydrodiol by microsomal epoxide hydrolase (mEH) that could then serve as a substrate for aflatoxin-aldehyde reductase (Ellis *et al.*, 1993). However, while early studies (Ch'ih *et al.*, 1983a,b) support a role for mEH in AFB detoxification, subsequent direct biochemical and kinetic studies (Guengerich *et al.*, 1996; Johnson *et al.*, 1997b; Wilson *et al.*, 1997) suggest no role for mEH in AFB metabolism. As water will rapidly and spontaneously react with *exo*-AFBO ($t_{1/2}$ 1 s) (Johnson *et al.*, 1996), enzymatic hydrolysis by mEH has been presumed to be insignificant. However, an epidemiological study by McGlynn *et al.* (1995) reported a statistical association between risk of HCC and occurrence of a polymorphic variant of mEH with somewhat lower activity due to protein destabilization (Hassett *et al.*, 1994; Omiecinski *et al.*, 2000). Previous epidemiological studies have found that polymorphic variants of mEH are associated with differences in risk for several types of cancer in individuals exposed to polycyclic aromatic hydrocarbons (PAHs). Unlike metabolism of AFB by mEH, the role of mEH in PAH metabolism involves generation of more reactive species such as benzo[*a*]pyrene-diolepoxide. Cortessis *et al.* (2001) found that a high-activity allele of mEH was associated with increased risk of colorectal adenoma in cigarette smokers, while Zhou *et al.* (2001) found that the low-activity allele examined in the McGlynn *et al.* (1995) study was a protective factor against lung cancer in heavy smokers and a risk factor in nonsmokers. Thus, the idea that differences in mEH activity

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may alter the development of xenobiotic-induced cancer is reasonable based on human population studies.

Because it is possible that enzymatic hydrolysis could be important in the lipid microenvironment, where the reactive epoxide is formed in the intact cell, we sought to re-examine the role of mEH in AFB genotoxicity by heterologous expression in the eukaryotic organism, *S. cerevisiae*. By reproducing the enzymatic pathway of bioactivation by human CYP1A enzymes coupled with coexpression of mEH, we could directly examine mEH function without membrane reconstitution, because both CYP1A and mEH enzymes colocalize in yeast endoplasmic reticulum (Eugster and Sengstag, 1993). In this study we were able to show that mEH does have a functional role in AFB detoxification, as measured by DNA adduct formation, mitotic recombination, and Ames assay mutagenicity. Our data provide an example of the strength of a eukaryotic yeast expression system for studying the role of biotransformation enzymes in the production and elimination of reactive intermediates in intact eukaryotic cells.

MATERIALS AND METHODS

Chemicals and enzymes. AFB and AFM were purchased from Sigma Chemical Co. (St. Louis, MO). [3H] AFB (16 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). HPLC grade solvents were purchased from J. T. Baker (Philipsburg, NJ). Yeast synthetic and complete media were purchased from BIO 101 (Vista, CA). Acrylamide and Bradford reagent were purchased from BioRad (Hercules, CA). The CYP1A antibody was supplied by Oxford Biomedical Research (Oxford, MI), and the mEH antibody was a generous gift from Dr. Curtis Omiecinski, University of Washington. Alkaline phosphatase-coupled secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Chemiluminescent substrate CSPD was purchased from Tropix (Bedford, MA). Restriction enzymes were all purchased from New England Biolabs (Beverly, MA).

Yeast strains. The parental *S. cerevisiae* strain YHE2 (*MATa/MATα, ade2-40/ade2-119, trp5-12/trp5-27, ilv1-92/ilv1-92, ura3Δ5/ura3Δ5*) and the transformed strains expressing human cDNAs have been previously described (Eugster and Sengstag, 1993; Eugster *et al.*, 1990) (except for pHE13 and pEK30) and are listed as follows: pDP34-control strain with no cDNA; pHE5-mEH; pHE10-CYP1A1; pHE12-CYP1A1/mEH coexpression; pHE36-CYP1A2; pHE13-CYP1A2/mEH coexpression; and pEK30-CYP1A2/ΔmEH. The yeast were transformed by standard methods (Schiestl and Gietz, 1989) and cultured in minimal synthetic media lacking uracil.

Plasmid constructs. All the plasmids used in this study have been described (Eugster and Sengstag, 1993) except for pHE13 and pEK30. For pHE13, the vector containing mEH (pHE5) was linearized with Sac I and an expression cassette for CYP1A2 was excised from pHE36 as a 2-kb Sac-I fragment and ligated into the Sac I-site of pHE5. The plasmid pEK30 is identical to pHE13 except that residue 226 of mEH was mutagenized from aspartic acid to glycine; a mutation known to abolish activity in rat mEH (Arand *et al.*, 1999). The single base-pair change was introduced by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA) and the following primer, CATTCAAGGAGGGGgCTGGGGGTCCC, and its complement (mutant base shown in lower case). The mEH expression cassette was excised from pHE5 as a 2.2 Kb Sac I/Sal I fragment and ligated into these same sites in pBluescript (Stratagene). After site-directed mutagenesis, the entire cassette was resequenced to verify the presence of the mutation and absence of any other nucleotide changes. The mEH cassette was then ligated into the Sac I/Sal I sites of pDP34. The CYP1A2 cassette was then cloned into the Sac I site as previously described for pHE13.

Yeast microsomes. Microsomes were prepared as previously described (Sengstag and Würgler, 1994) and aliquots snap frozen in liquid nitrogen and stored at −80°C prior to use. Protein concentrations were determined with Bradford reagent (BioRad) with bovine serum albumin as the standard. Heterologous microsomal P450 content was measured by the method of Omura and Sato (1964).

Enzyme assays. The catalytic activity of the microsomes towards AFB was determined as described by Gallagher *et al.* (1994). Briefly, approximately 1 mg of yeast microsomal protein, 500 μg of a 2:1 (v:v) mixture of rat:mouse hepatic cytosol (to quantitatively capture the reactive AFB-epoxide as the glutathione conjugate, Gallagher *et al.*, 1994), 1 mM NADPH and 5 mM reduced glutathione in 0.1 M potassium phosphate, pH 7.2 were mixed in a total volume of 240 μl. After a 5-min preincubation at 37°C, the reaction was started by the addition of 10 μl of AFB dissolved in dimethysulfoxide, for a final concentration of 128 μM as determined by UV spectrophotometric analysis of stock solutions (Busby and Wogan, 1984). The reactions were allowed to proceed at 37°C for 10 min and then terminated by addition of 250 μl ice-cold methanol containing 1% trifluoroacetic acid and AFG_1 (10 μM) as an internal standard. AFB metabolites were separated by reversed-phased, high-performance liquid chromatography as previously described (Monroe and Eaton, 1987) and measured by UV detection. Microsomal epoxide hydrolase activity for cis-stilbene oxide (CSO) was performed as described by Gill *et al.* (1983) with the following modifications. Yeast microsomes were diluted in 10 mM potassium phosphate buffer, pH 7.4 for a final protein concentration of 1 mg/ml in a 100 μl reaction volume. The microsomes were incubated for 5 min at 37°C and then 1 μl of [^{3}H] CSO stock solution was added for a final concentration of 50 μM [^{3}H] CSO (specific activity ~10 mCi/mmol). After 20 min at 37°C, the reactions were stopped by addition of 200 μl iso-octane followed by vortexing; aliquots of the aqueous phase were analyzed by liquid scintillation counting to quantitate the diol product.

Immunoblotting. Yeast whole-cell extracts or microsomal protein fractions were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA). Nonspecific binding was blocked with 5% BLOTTO dissolved in phosphate-buffered saline containing 0.1% TWEEN 20. Primary antibodies against either human mEH or rabbit CYP1A were diluted in blocking buffer 1:15,000 or 1:100, respectively. Binding of antibody to antigen was detected with alkaline phosphatase-conjugated secondary antibody and visualized by exposure to *X-Omat* AR™ film (Eastman Kodak, Rochester, NY) using chemiluminescent CSPD substrate.

Trp recombination test. The homologous recombination between the *trp5-12/trp5-27* alleles was performed as previously described (Sengstag and Würgler, 1994). Briefly, yeast were grown to the exponential phase, washed in sodium phosphate buffer (100 mM, pH 7.4) and then resuspended in buffer at a concentration of 10^8 cells/ml. Cells were then exposed to AFB or AFM in DMSO at a final concentration of 5% v/v DMSO for 4 h at 30°C, centrifuged and washed twice with tryptophan-deficient media before plating on agar plates lacking tryptophan. Trp^+ convertants were scored after 3 days of growth at 30°C. The conversion frequency was calculated by comparing it to the number of colonies that grew on complete (YPD) media plates (after diluting the cell cultures 1:40,000 in media, to obtain plating densities easily counted).

AFB-DNA adduct formation. Exponentially growing yeast were harvested and 0.05 OD units were inoculated into 0.5 ml 100 mM sodium phosphate buffer, pH 7.4 containing [^{3}H] AFB (specific activity 18.6 Ci/mmol) dissolved in methanol for a final solvent concentration of 1.7% (v:v). The cells were incubated at 30°C for 6 h, protoplasts prepared as described (Sengstag and Würgler, 1994), and then total nucleic acid was isolated by alkaline lysis followed by ethanol precipitation. Cellular RNA was removed by treatment with 0.2 mg/ml RNase A and then extracted with phenol/chloroform, followed by ethanol precipitation. The DNA was quantified by UV absorption and adducts were measured by liquid scintillation counting.

Ames assay. The *Salmonella typhimurium* strain TA98 (*hisD3052, rfa,* Δ*uvrB,* pKM101) was used to assay the mutagenicity of AFB metabolized by yeast microsomes as described previously (Sengstag and Würgler, 1994). In

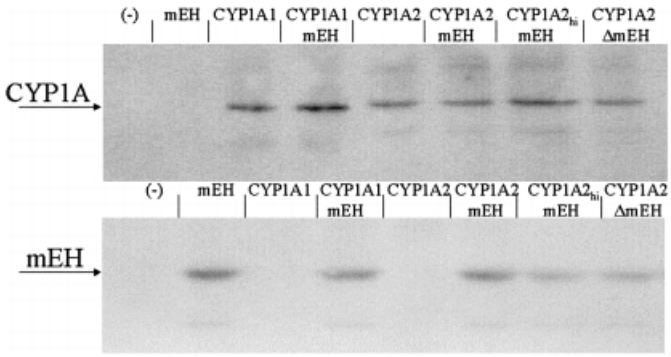

FIG. 1. Western-blot analysis of heterologous expression in purified microsomes. Negative control microsomes are in the first lane followed by the indicated cDNA expressing strains. CYP1A2$_{hi}$/mEH represents the strain with approximately doubled expression of CYP1A2 relative to CYP1A2 or CYP1A2/mEH while CYP1A2/ΔmEH is the mutated form of mEH lacking any catalytic activity. Duplicate gels were run for the mEH antipeptide antibody and polyclonal CYP1A antibody and each lane contains 20 μg of microsomal protein.

brief, an overnight culture of TA98 was harvested and resuspended in 150 mM potassium chloride, pH 7.4; 100 μl of bacteria were then added to 700 μl of an NADPH-regenerating system (containing 150 mM potassium phosphate, pH 7.4, 0.75 mM NADP+, 15 mM glucose 6-phosphate, 10 mM magnesium chloride and 10 units glucose 6-phosphate dehydrogenase), followed by 200 μg of yeast microsomes and subsequent addition of 30 μl of AFB. The cell mixture was incubated for 20 min at 37°C before 2 ml of top agar was added and the mixture poured onto minimal plates. The revertant colonies were then scored after incubation at 37°C for 2 days.

Statistical analysis. All data are presented as mean ± SEM and are derived from triplicate cultures of at least duplicate experiments. Results were compared by one-way ANOVA and Tukey-Kramer multiple comparisons test, using the statistical program InStat for the Macintosh (Graphpad Software Inc., San Diego, CA). A $p \leq 0.05$ was considered significant.

RESULTS

The expression profiling for the recombinant yeast strains used in this study are shown by Western blot in Figure 1. Microsomal proteins (20 μg/lane) were separated by SDS–PAGE on duplicate 10% polyacrylamide gels and then probed with either an antibody raised against rabbit CYP1A protein from 3-methylcholanthrene induced liver or an antipeptide antibody raised against human mEH (amino acids 42–60). The level of immunoreactive CYP1A1 protein was slightly higher in CYP1A1/mEH coexpressor compared to CYP1A1 alone, while CYP1A2 was uniform except for one clone, designated CYP1A2$_{hi}$/mEH, which had somewhat higher levels. The mEH protein levels exhibited some variation between strains with the site-directed inactive mutant of mEH (CYP1A2/ΔmEH) having lower amounts of immunoreactive mEH protein. However, this makes little practical difference because the protein lacks any catalytic activity. In addition to determining expression by Western blot, we measured heterologous P450 content and specific activity towards AFB for microsomes purified from recombinant yeast strains. The yeast strain transformed with the vector containing no human cDNAs gave no signal by CO difference spectra nor was there measurable AFB-oxidizing activity in microsomes purified from this strain. While Western-blot analysis suggested differences in CYP1A1 content, the specific activity towards AFB, as measured by formation of the hydroxylated metabolite aflatoxin M_1 (AFM), was similar (Table 1). Likewise, P450 content did not significantly differ between yeast expressing CYP1A1 alone or in conjunction with mEH. For yeast expressing CYP1A2, specific activity towards AFB for generation of either AFM or AFBO (as measured by the glutathione-conjugate AFB-GSH) were similar for all the strains, with the exception of a single clone of CYP1A2/mEH coexpressor that had approximately twice as much P450 content as measured by CO difference spectra. This particular clone is referred to as CYP1A2$_{hi}$/mEH. It should be noted that several other clones of CYP1A2/mEH were analyzed and none of them had levels as high as CYP1A2$_{hi}$/mEH but were similar to CYP1A2 or CYP1A2/mEH clones. When

TABLE 1
Analysis of Heterologous P450 Content, AFB Metabolism, and CSO Activity of Yeast Microsomes Expressing Human CYP1A and mEH Enzymes

	CYP1A1	CYP1A1 (mEH)	CYP1A2	CYP1A2 (mEH)	CYP1A2$_h$ (mEH)	CYP1A2 (ΔmEH)
AFM[a]	402 ± 39	368 ± 29	49 ± 5	48 ± 5	75 ± 6	52 ± 4
	(10,993 ± 1066)	(10,595 ± 833)	(3525 ± 36)	(3750 ± 39)	(3086 ± 16)	(3939 ± 30)
AFBO[a,b]	1.8 ± 0.5	2.6 ± 1.0	46 ± 2	44 ± 2	78 ± 2	51 ± 2
	(50 ± 14)	(74 ± 29)	(3303 ± 140)	(3458 ± 170)	(3207 ± 79)	(3864 ± 120)
CSO hydrolysis[c]	<0.2	4.7 ± 0.3	<0.2	5.3 ± 0.9	4.9 ± 0.8	<0.2
P450 content[d]	36.6 ± 4.3	34.8 ± 3.7	13.9 ± 0.8	12.8 ± 1.2	24.3 ± 4.3	13.2 ± 3.0

[a]Reaction rates were determined at a substrate concentration of 128 μM AFB and are given as pmol/min/mg microsomal protein; the value in parentheses is pmol/min/nmol P450. Control yeast microsomes had no detectable AFB oxidizing activity.

[b]The epoxide AFBO was measured by trapping the glutathione-conjugate with a mixture of rat and mouse liver cytosolic GST enzymes as described (Gallager *et al.*, 1994).

[c]Reaction rates were determined with 50 μM [^{3}H] CSO and are given as nmol diol product/min/mg protein.

[d]P450 content was determined by CO difference spectra and presented as pmol P450/mg microsomal protein; control yeast microsomes had no detectable P450. The values for CYP1A/mEH enzymatic activity and P450 content are given as the mean ± SEM.

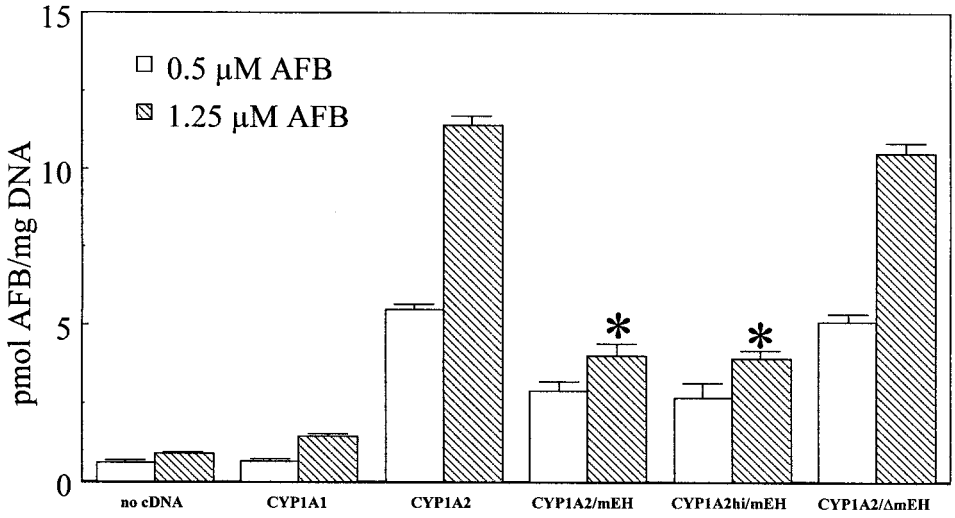

FIG. 2. AFB-DNA adduct formation in yeast expressing CYP1A2 and attenuation by mEH. Cells were exposed to [^{3}H] AFB_1, genomic DNA was isolated and adduct levels quantified by liquid scintillation counting. Yeast expressing CYP1A1 had adduct concentrations comparable to control yeast while CYP1A2-expressing yeast had adduct concentrations significantly greater than background. *Coexpression of mEH blocked DNA adduction with significant effect ($p < 0.05$) at 1.25 μM AFB. Data are mean ± SEM from samples analyzed in triplicate.

specific enzymatic activities are expressed in terms of reaction rates per nmol P450 instead of per mg microsomal protein this difference disappears. While it is difficult to directly compare cytochrome P450 content and enzymatic activity of CYP1A2 heterologously expressed in yeast with that observed in human liver, it is worth noting that our yeast P450 content, in terms of pmol/mg microsomal protein, is within the range observed for human liver microsomes (Guengerich, 1995). The enzymatic characteristics of yeast-expressed human mEH towards carbamazepine epoxide have been previously described (Eugster *et al.*, 1991) and are similar to what has been observed for human liver microsomes. In addition, specific activity towards cis-stilbene oxide (CSO) was similar for all mEH-expressing strains with the exception of CYP1A2/ΔmEH that lacked any activity (Table 1). However, caution must be used when trying to draw direct comparisons, since these values are expressed in terms of reaction rate per mg microsomal protein. Nevertheless, as with CYP1A2, mEH expression and activity is not significantly different from human liver; taking these 2 values (CYP1A2 and mEH) together would suggest that the ability of yeast microsomes to activate and hydrolyze AFB might be within the range seen in human liver.

To determine if mEH expression could offer any protection against AFB genotoxicity, we analyzed formation of AFB-DNA adducts in exponentially growing yeast exposed to [^{3}H] AFB. As seen in Figure 2, expression of CYP1A1 did not promote adduct formation over background, consistent with our previous observations that CYP1A1 preferentially forms AFM and only small amounts of AFBO. Expression of CYP1A2 resulted in a significant increase of adduct levels over background with a dose-dependent increase in adduct levels (5.5 and 11.4 pmol AFB/mg DNA at 0.5 and 1.25 μM AFB, respectively, Fig. 2). When cells expressing both CYP1A2 and mEH were exposed to [^{3}H] AFB, they too formed DNA adducts above background levels, but at 1.25 μM AFB, the levels of adducts were significantly lowered relative to CYP1A2 alone (4.0 and 3.9 pmol AFB/mg DNA, CYP1A2/mEH and $CYP1A2_{hi}$/mEH, respectively; $p < 0.05$).

To further analyze a possible role for mEH in AFB detoxification, we studied AFB genotoxicity by 2 separate methods. The parental yeast strain used for heterologous expression is diploid and auxotrophic for the amino acid tryptophan due to mutations in the *trp5* alleles. If these 2 alleles undergo mitotic recombination, gene conversion may occur, allowing yeast to grow in the absence of tryptophan. As previously described (Sengstag and Würgler, 1994) and shown in Figure 3, yeast expressing CYP1A1 or CYP1A2 undergo gene conversion in response to AFB exposure in a dose-dependent fashion. When cells expressing either CYP1A1 and mEH or CYP1A2 and mEH were exposed to AFB, they too exhibit gene conversion in a dose dependent manner, but at significantly lower levels when compared to strains expressing CYP1A1 or CYP1A2 alone (Fig. 3). Furthermore, the inactive mEH mutant was unable to confer any protection against AFB genotoxicity as measured by *trp5* gene conversion, indicating that enzymatic activity, rather than some unrelated event associated with mEH protein expression, is responsible for the reduction in mitotic recombination. However, if binding to the mEH protein, rather than catalytic activity, were responsible for the protective effect of expressed mEH, the null-mEH control experiment might have underestimated a protective effect in the CYP1A2/ΔmEH construct, because Western-blot analysis indicated that there was somewhat less inactive mEH protein expressed in the

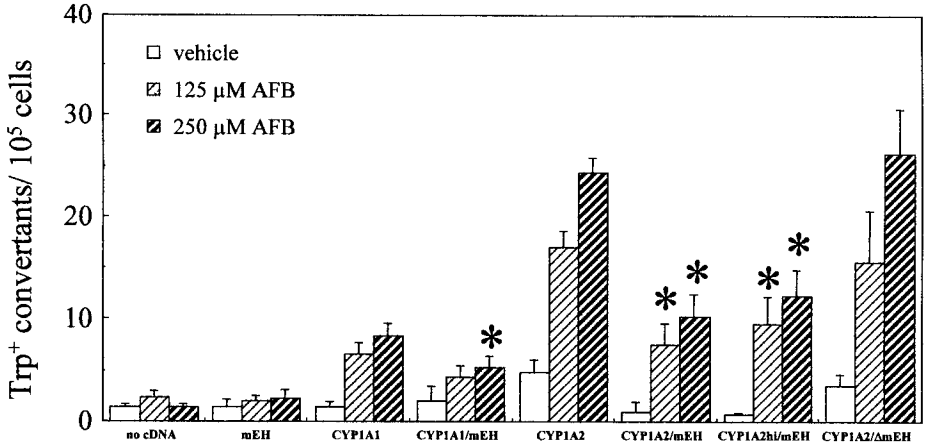

FIG. 3. Modulation of AFB genetic toxicity assessed by mitotic gene conversion at the *trp5* locus in intact yeast. Yeast were exposed to the indicated concentrations of AFB in culture and then plated on tryptophane deficient medium to select for recombinants while plating efficiency/survival was assessed by plating on complete medium. Intracellular biotransformation of AFB by either CYP1A1 or CYP1A2 resulted in a dose-dependent increase in mitotic recombination and coexpression with mEH and either P450 attenuated this response. Data are mean ± SEM from samples of a representative experiment analyzed in triplicate.

CYP1A2/ΔmEH construct, relative to the CYP1A2/mEH construct (Fig. 1).

The role of mEH in AFB genotoxicity was also examined by Ames assay. When microsomes were isolated from recombinant strains and used as an activating system in the Ames assay with tester strain TA98, both CYP1A1- and CYP1A2-containing microsomes were able to cause a dose-dependent increase in frame-shift mutations as scored by recovery of histidine revertants (Fig. 4). Similar to what was observed with *trp5* gene conversion, coexpression of mEH with either CYP1A1 or CYP1A2 resulted in significant protection against AFB mutagenicity, and the catalytically inactive mutant of mEH conferred no protection. For both measures of genotoxicity, mEH was able to afford a greater degree of protection in cells expressing CYP1A2, as compared to cells expressing CYP1A1. This is not surprising, however, because of the metabolite profiles of CYP1A2 and CYP1A1. CYP1A1 metabolizes AFB almost exclusively to AFM with only minimal AFBO generation, whereas CYP1A2 forms an approximate 1:1 ratio of AFM and AFBO (Table 1). The carcinogenicity of AFM in rats is approximately 10% of AFB (Hsieh *et al*., 1984) and is generally considered to be a route of detoxification. The finding that CYP1A1-expressing yeast are able to convert AFB to a genotoxic metabolite in both the Ames and *trp*5 gene conversion assays suggests that AFM is a potent mutagen. To investigate this further, we exposed control and recombinant

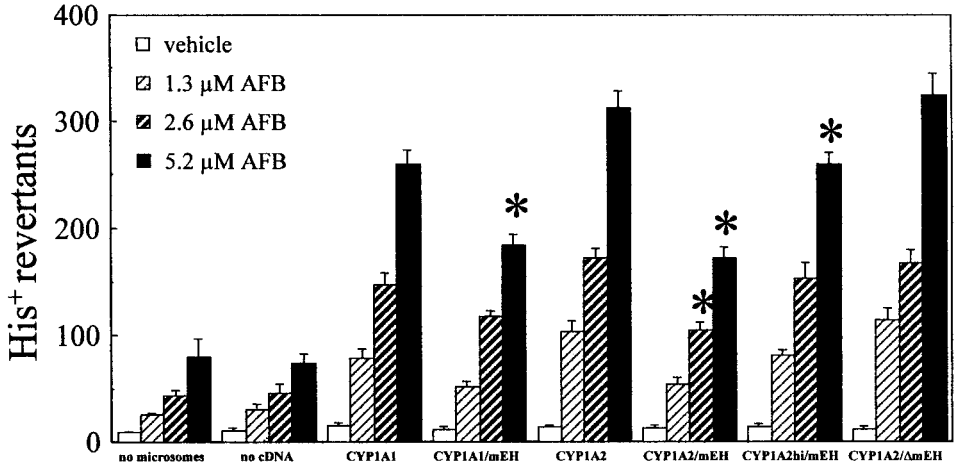

FIG. 4. Activation of AFB by recombinant yeast microsomes to a mutagenic compound in the Ames assay. *S. typhimurium* tester strain TA 98 was incubated with AFB and microsomes from the indicated strains as described in Materials and Methods and His^+ revertants were scored. CYP1A-containing microsomes exhibited a dose-dependent increase in AFB mutagenicity, which was decreased by mEH. Statistically significant differences ($p < 0.05$) for CYP1A1 or CYP1A2 alone versus the respective CYP-containing strain coexpressing mEH are indicated by (*). Data are mean ± SEM.

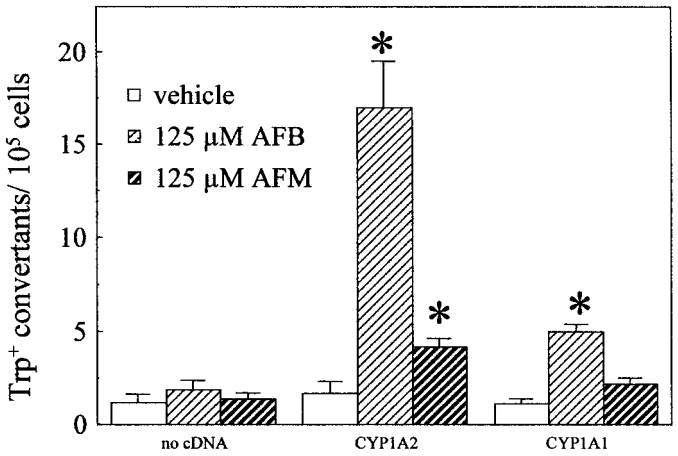

FIG. 5. Comparison of mitotic recombination induced by AFB or AFM. Yeast was exposed to equal concentrations of either AFB or AFM and then mitotic recombination scored by reversion of tryptophan auxotrophy. AFM induced mitotic recombination in cells expressing either CYP1A1 or CYP1A2 to levels above background but the values were not statistically significant. Statistically significant differences ($p < 0.05$) between yeast expressing no cDNA versus CYP1A1 or CYP1A2 are indicated by an (*). Data are mean ± SEM from 2 separate experiments, each analyzed in triplicate ($n = 6$).

yeast directly to AFM and measured mitotic recombination rates by *trp*5 gene conversion in comparison to AFB. As seen in Figure 5, AFB was significantly more active at inducing mitotic recombination relative to AFM. In addition, AFM was not able to induce mitotic recombination in the absence of the CYP1A enzyme. The ability of yeast-expressed CYP1A enzymes to bioactivate AFM was also investigated by incubating AFM with purified microsomes and analyzing metabolite production by HPLC. However, neither CYP1A2 nor CYP1A1 appeared capable of converting AFM to the corresponding epoxide or other oxidation products (data not shown).

DISCUSSION

In this study, we have demonstrated, for the first time, that mEH has the ability to confer protection against AFB genotoxicity, as measured by DNA-adduct formation, mitotic recombination and Ames assay mutagenicity in doubly transfected yeast. Although previous studies have failed to demonstrate a protective role of human mEH in AFB-induced genotoxicity (Guengerich *et al.*, 1996; Johnson *et al.*, 1997b; Wilson *et al.*, 1997), there are substantial methodological differences between this study and the previous studies. In this study, we investigated mEH function in an intact cell system where, with the exception of the Ames assay, the reactive metabolite AFBO was generated *in situ*, where both CYP and mEH enzymes colocalized to the same subcellular compartment. In the study of Wilson *et al.* (1997), the researchers were investigating sister chromatid exchange (SCE) in peripheral lymphocytes exposed to AFB and human liver microsomes of varying CYP1A2 and mEH phenotype (i.e., supposed varying activation and detoxification activities, respectively). Perhaps SCE is not a very reliable measure of AFB genotoxicity, given the fact that they saw only a very modest protection (~2-fold decreased genotoxicity) in lymphocytes exposed to AFB and mouse liver microsomes in the presence or absence of mouse liver cytosol. The presence of mGstA3-3 in mouse liver cytosol would be expected to significantly decrease AFB genotoxicity but this was not observed. Since we believe that mEH has only a modest effect on AFB genotoxicity, and mGstA3-3 is capable of greatly reducing AFB genotoxicity, it is not surprising that Wilson *et al.* were unable to find an association between mEH phenotype (based on CSO activity) and rates of SCE.

The kinetic studies of Guengerich *et al.* (1996) were conducted with bacterially expressed CYP3A4 and purified rat or human mEH that were subsequently reconstituted *in vitro*. This study found that only at very high ratios of mEH to CYP3A4 could mEH alter AFB genotoxicity as assessed by *umu* response in *S. typhimurium* tester strain TA1535. The experiments by Johnson *et al.* (1997b) found that the rate of hydrolysis of AFBO was unaffected by addition of purified human mEH while purified rat mEH was able to slightly increase the hydrolysis rate.

Significant differences exist between those experiments and this current study. First, we are using CYP1A2, not CYP3A4, and in our case the enzyme-coding sequence (i.e., deletion of N-terminal membrane anchor) has not been altered to facilitate expression in *E. coli*; however, these differences are not likely to be of major importance. Second, we are expressing mEH and CYP1A2 together so they colocalize without any membrane reconstitution utilizing synthetic lipids. Finally, in our recombinant yeast, the AFBO is generated in the endoplasmic reticulum (a hydrophobic environment) which may protect the CYP-generated AFBO from non-enzymatic hydrolysis.

Because mEH and CYP enzyme are localized to the same subcellular compartment, mEH may well be positioned to facilitate hydrolysis of AFBO near its intracellular site of formation. Although it is conceptually difficult to understand how the hydrophobic environment of the endoplasmic reticulum could effectively protect CYP-generated exo-AFBO from the high concentration of intracellular water present in the intact cell or sub-cellular fractions, this must indeed occur, because numerous studies have previously demonstrated the effectiveness of certain glutathione *S*-transferases with high efficiency toward AFBO to effectively compete with water for the highly reactive *exo*-AFBO (Buetler *et al.*, 1992; Ch'ih *et al.*, 1983b; Guengerich *et al.*, 1996; Raney *et al.*, 1992a,b; Gallagher *et al.*, 1994; Johnson *et al.*, 1997a). Thus, in the absence of an effective glutathione conjugation system such as occurs in the mouse, and to a lesser extent in rat liver, mEH activity may play a functional role in protection of DNA against the reactive AFB-exo-8,9-oxide. Because primate GSTs, including human, have very low AFBO-conjugating activity (Raney *et al.*, 1992b; Johnson *et al.*, 1997a; Wang *et al.*, 2000) relative to that seen in rodent liver (Buetler *et al.*,

1992; Van Ness *et al.*, 1998), mEH may provide important protection against AFB-induced DNA damage in the human liver.

The observation that CYP1A1 was capable of converting AFB to a mutagenic metabolite in both intact yeast (*trp*5 gene conversion) and in purified microsomes (Ames assay) is puzzling since CYP1A1 forms AFM at a high rate but generates little AFBO and forms no detectable DNA adducts. However, the acute toxicity of AFM in rats is actually close to what one sees with AFB (Pong and Wogan, 1971). We tested the possibility that CYP1A1 or an endogenous enzyme is further metabolizing AFM to a genotoxic compound. Exposure of yeast to purified AFM only induced a slight increase in *trp*5 gene conversion in CYP1A-expressing cells while incubation of AFM with microsomes expressing CYP1A1 or CYP1A2 yielded no detectable metabolites. The low rate of mitotic recombination may be a result of poor permeability of AFM through the yeast cell wall. Previously, it was shown that human liver microsomes were capable of bioactivating AFM to the corresponding epoxide (Neal *et al.*, 1998). In contrast to these findings, we were unable to detect such a metabolite, either with yeast-expressed CYP1A1 or CYP1A2 or with several different human liver microsome preparations. The reasons for this are not clear but may be due to sensitivity of the assay. The fact that CYP1A1 is capable of forming a mutagenic metabolite (as assessed by Ames assay mutagenicity and reversion of tryptophan prototrophy) and that mEH attenuates this response would seem to imply that an epoxide intermediate is involved, although quantity is very small, relative to the amount of AFM1 formed, and is below the limit of detection of our HPLC-based assay.

In conclusion, we have provided several lines of evidence that coexpression of mEH is capable of modifying the genotoxic properties of AFB, thus supporting the epidemiological correlation that differences in mEH activity due to genetic polymorphisms can alter the risk of AFB-induced hepatocellular carcinoma (McGlynn *et al.*, 1995). The use of recombinant yeast to recreate a multi-step biotransformation pathway facilitated this finding in a manner not utilized in previous studies, which found no significant role for mEH in AFB metabolism. Finally, because human gene products are readily expressed and function in yeast in a manner similar to higher organisms, they provide a useful system for testing the functional significance of sequential human biotransformation enzymes involved in multistep xenobiotic activation and detoxification.

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