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Heterocyclic aromatic amines efficiently induce mitotic recombination in metabolically competent *Saccharomyces cerevisiae* strains

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Heterocyclic aromatic amines (HAs) represent a class of potent bacterial mutagens and rodent carcinogens which gain their biological activity upon metabolic conversion by phase I and phase II enzymes. Subsequent to cytochrome P450 (CYP)-dependent hydroxylation, mainly catalyzed by CYP1A2, acetylation mediated by the activity of *N*-acetyltransferase, NAT2, produces the ultimate electrophilic product that may react with DNA. In addition to point mutations observed in HA-exposed cells as genotoxic endpoint *in vitro*, loss of heterozygosity (LOH) has often been identified in HA-related rodent tumors as another endpoint *in vivo*. LOH may reflect a chromosomal deletion, a chromosome loss or a previous mitotic recombination event and it represents a prominent mechanism for the inactivation of tumor suppressor alleles. In this study we have investigated whether LOH observed in several HA-induced rodent tumors is related to a recombinogenic activity of HA compounds, and to address this question we have studied the genotoxic activity of several HAs in metabolically competent *Saccharomyces cerevisiae* strains. For this purpose expression vectors have been constructed providing simultaneous expression of three human enzymes, CYP1A2, NADPH-cytochrome P450 oxidoreductase and NAT2 in different genotoxicity tester strains. Evidence for functional expression of all three enzymes has been obtained. One strain allowed us to monitor HA-induced gene conversion, another one HA-induced chromosomal translocation. A third strain allowed us to study HA-induced forward mutations in the endogenous *URA3* gene. It was found that 2-amino-3-methylimidazo-[4,5-f]quinoline and 2-amino-3,8-dimethylimidazo-[4,5-f]quinoxaline produced a strong recombinogenic response in either recombination tester strain. The recombinogenic activity was comparable with the mutagenic activity of the compounds. The other HAs, 2-amino-3, 4-dimethyl-imidazo-[4,5-f]quinoline, 2-amino-6-methyldipyrido-[1,2-a:3′,2′-d]imidazole, 2-aminodipyrido-[1,2-a:3′, 2′-d]imidazole, 3-amino-1-methyl-5H pyrido-[4,3-b]indole and 2-amino-1-methyl-6-phenyl-imidazo-[4,5-b]pyridine, produced weak or no increases in the genotoxic endpoints of interest. The described strains may provide a suitable tool to characterize the genotoxic potential of HAs in more detail.

Abbreviations: CYP, cytochrome P450; 5-FOA, 5-fluoro-orotic acid; Glu-P-1, 2-amino-6-methyldipyrido-[1,2-a:3′,2′-d]imidazole; Glu-P-2, 2-aminodipyrido-[1,2-a:3′,2′-d]imidazole; HA, heterocyclic aromatic amine; hOR, human NADPH-cytochrome P450 oxidoreductase; IQ, 2-amino-3-methylimidazo-[4,5-f]quinoline; LOH, loss of heterozygosity; MeIQ; 2-amino-3,4-dimethyl-imidazo-[4,5-f]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo-[4,5-f]quinoxaline; NAT, *N*-acetyltransferase; OAT, *O*-acetyltransferase; PhIP, 2-amino-1-methyl-6-phenyl-imidazo-[4,5-b]pyridine; SMZ, sulfamethazine; Trp-P-2, 3-amino-1-methyl-5H pyrido-[4,3-b]indole.

Introduction

A large number of heterocyclic aromatic amines (HAs) have been identified in cooked, broiled and fried meat and fish over the past years (1,2; reviewed in refs 3,4). Several of these compounds have been classified as potent mutagens in the Ames assay using *Salmonella typhimurium* (1,5) and have also been shown to act as carcinogens in rodents and monkeys, inducing tumors predominantly in the liver as well as in other organs and tissues (6; reviewed in refs 7–10).

HAs require metabolic activation by drug metabolizing-enzymes to exert their mutagenic and carcinogenic effects (11). The initial step involves *N*-hydroxylation catalyzed mainly by cytochrome P450 (CYP)1A2 (12–14), followed by *O*-acetylation catalyzed by *N*,*O*-acetyltransferase, NAT2 (15–17) or *O*-sulfonation catalyzed by sulfotransferase (18). The resulting *N*-acetoxyesters or *N*-sulfoxyesters readily react with protein and DNA via formation of arylnitrenium ions (11).

The major DNA adducts formed by activated HAs *in vitro* and *in vivo* have been identified as *N*-(deoxyguanosin-8-yl)-HA adducts (19,20) and to a lesser extent, 5-(deoxyguanosin-N^2-yl)-HA adducts (21,22), premutagenic lesions which give rise to various types of molecular alterations. In *S.typhimurium* HAs preferentially induce frameshift mutations, primarily CG deletions detected as reversion of the *his*D3052 allele (23,24). At higher concentrations, base substitutions, predominantly GC→TA transversions, and to some extent, also GC→AT transitions have also been detected in the *hisG46* allele when revertants were analyzed by hybridization with mutation-specific oligonucleotides (25). Similarly, using a set of mutant *lacZ* alleles located on F′ episomes in *Escherichia coli*, Watanabe and Ohta (26) showed that >99.5% of HA-induced mutations were –2 frameshift mutations, whereas only a small amount were GC→TA transversions. Recently, Broschard *et al*. (27) characterized 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ)-induced forward mutations in the yeast *URA3* gene as target located on a ColE1-derived plasmid. The system comprised *in vitro* mutagenesis of the plasmid with chemically activated IQ followed by transformation into *E.coli*. It was found that the specific nature of genetic alteration depended on the extent of base modification: at low ratios of IQ adducts/plasmid base substitutions, mostly GC→AT transitions (~70%) predominated, whereas at higher plasmid modification levels, complex DNA rearrangements were induced preferentially.

In mammalian cell systems HAs predominantly induce base substitutions. Forward mutations selected in the bacterial *supF* target gene present in shuttle vector pSP189 that had replicated in HA exposed human fibroblasts revealed a high fraction (85–93%) of base substitutions, predominantly GC→TA transversions (28). In addition to other base substitutions, such GC→TA

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transversions were also the major alterations observed at the *hprt* locus of V79 Chinese hamster cells or human lymphoblastoid TK6 cells exposed to the metabolically activated HA-compound 2-amino-1-methyl-6-phenyl-imidazo-[4,5-b]pyridine (PhIP; 29,30). Apart from base substitutions, frameshift mutations involving the loss of a single GC pair have been found. Interestingly, analysis of PhIP- and IQ-induced colon tumors in male rats revealed that four out of eight had an identical –1 frameshift mutation resulting in a GC base pair deletion in a 5′-GGGA-3′ sequence of the *Apc* tumor suppressor gene, whereas in 13 IQ-induced tumors only base substitutions were found (31).

Although point mutations have been repeatedly identified in cancer-related genes, such as H-*ras*, *p53* and *Apc* (32–34) from HA-induced experimental tumors, still other genetic alterations have emerged. These comprised the destabilization of microsatellite sequences as well as loss of heterozygosity (LOH), reflected by the complete loss of specific alleles (32,34–36).

The major consequence of LOH is the loss of heterozygous alleles, and by loss of the wild-type allele of a gene a previously masked mutant allele may be uncovered. Apart from chromosomal non-disjunction and large deletion, mitotic recombination has been suggested as an important underlying mechanism leading to LOH (37; reviewed in ref. 38). Indeed, a mitotic recombination event occurring in G_2 phase of the cell cycle between homologous chromosomes will lead to homozygosity of all markers distal from the crossing-over site in 50% of the mitotic divisions. Thus, any activity of a given chemical to induce mitotic recombination may contribute to LOH and thereby lead to allelic loss, e.g. of heterozygous tumor suppressor genes. Since a considerable fraction of HA-induced rodent tumors exhibited LOH we addressed the question of whether a recombinogenic activity of HA compounds could be detected apart from their mutagenic activity and how the two activities compared with each other quantitatively.

Xenobiotic-induced mitotic recombination can be easily identified in the lower eukaryote *Saccharomyces cerevisiae*. Various strains are available to monitor distinct recombination related events like gene conversion (39,40) and translocation (41). Moreover, various human cDNAs coding for drug metabolizing enzymes that are required for the activation of polycyclic aromatic compounds have been expressed in these strains (40,42–45). This strategy solved the problem of specific metabolic incompetence of the *S.cerevisiae* strains. Moreover, the expression of human enzymes in the genotoxicity tester strains ensured the occurrence of human-specific metabolism of the compounds of interest, thus overcoming problems related to species specificity.

In this paper we describe the construction of *S.cerevisiae* strains actively co-expressing the cDNAs for human *CYP1A2*, NADPH-cytochrome P450 oxidoreductase (*hOR*) and *NAT2*, necessary for the activation of HAs to carcinogens. We observed that several HAs, upon metabolism to reactive products in yeast, induced mitotic recombination either detected as gene conversion or chromosomal translocation as well as forward mutations. Our newly established strains may represent a valuable tool to study HA metabolism and further characterize the genetic consequences of HA exposure.

Materials and methods

Chemicals

The HA compounds were purchased from Toronto Research Chemicals (Ontario, Canada) and dissolved in dimethyl sulfoxide. Sulfamethazine (SMZ) and acetylCoA were purchased from Sigma (Buchs, Switzerland), amino acids, Ade and Ura from Merck (Dietikon, Switzerland), yeast nitrogen base and bacto agar from Difco (Chemie Brunschwig, Basel), 4-dimethylamino-benzaldehyde from Fluka (Buchs, Switzerland) and 5-fluoro-orotic acid (5-FOA) from PCR Inc. (Gainesville, FL). DNA modifying enzymes and *Taq* DNA polymerase were obtained from Boehringer Mannheim (Mannheim, Germany). Thermostable DNA polymerase from *Thermococcus litoralis* (Vent DNA polymerase) was obtained from New England Biolabs (Beverly, MA).

Strains and media

Yeast transformants were grown on YM medium (0.67% yeast nitrogen base without amino acids, 2% glucose) containing, if required, the following supplements: Ade, Ura, Trp and His at 20 mg/l, Ile and Leu at 30 mg/l and Val at 150 mg/l. Ura^- mutants were selected on 5-FOA plates consisting of 6.7 g/l nitrogen base, 20 g/l bacto agar, 20 g/l glucose, 1 g/l FOA, 35 mg/l Ura, 20 mg/l Ade, His and Trp, 30 mg/l Leu and Ile, and 150 mg/l Val. YPD contained 1% yeast extract, 2% bacto tryptone and 2% glucose. *Escherichia coli* transformants were selected on LB or M9 medium (46) containing 100 mg/l ampicillin (Sigma).

Yeast strains

Strain YHE2 (*MAT*a/α, *ade2-40*/*ade2-119*, *ilv1-92*/*ilv1-92*, *trp5-12*/*trp5-27*, *ura3*Δ*5*/*ura3*Δ*5*) (40) was used to monitor allelic gene conversion between the heteroallelic trp5 genes by selection for Trp^+ subclones. Strain YB110 (*MAT*a/α, *ade2-101*/*ade2-101*, *ura3-52*/*ura3-52*, *his3-*Δ*200*/*his3-*Δ*200*, *leu2*/*LEU2*, *GAL1::his3-*Δ*5′*/*GAL1*, *trp1::his3-*Δ*3′*/*trp1-*Δ*1*, *LYS2*/*lys2-801*) (41) was used to quantitate chromosomal translocation by selection for His^+. Monitoring of mutations in *URA3* was performed with strain YGP1B (*MAT*a, *his3-11,15*, *ts1*). This strain was obtained upon a tetrad dissection of a diploid strain that resulted from the crossing of YS18 (*MAT*α, *his3-11,15*, *leu2-3*, *112*, *ura3*Δ*5*) (47) to strain D7-5Dts1 (*MAT*a, *ade2-40*, *trp5-12*, *ilv1-92*, *ts1*) (48) which was a generous gift of Prof. P.Venkov (Bulgarian Academy of Sciences, Sofia, Bulgaria) and contains the ts1 mutation which has been shown to increase the permeability of the cells towards various toxic compounds.

Bacterial strains

Escherichia coli strain DH5αF′ [*F′*, *endA1*, *hsdR17*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, ϕ*80dlacZ*Δ*M15*, Δ(*lacZYA argF*) U169] was used for plasmid constructions and transformation experiments. Strain MC1061 [*araD139*, Δ(*ara*, *leu*)*7697*, *lac*Δ*X74*, *galU*, *galK*, *hsdR2*, *strA*, *mcrA*, *mcrB1*] (49) or strain KC8 (*hsdR*, *leuB600*, *pyrF::Tn5*, *hisB463*, *lac*Δ*X74*, *strA*, *galU*, *K*) (50) was used for plasmid rescue from yeast.

Plasmid constructions

DNA manipulations were performed according to standard protocols (46). PCR amplification of DNA fragments was done in a 50 µl reaction volume containing 1× Vent buffer (New England Biolabs), 200 ng plasmid template, 1 µM each primer, 200 µM dNTPs, 1.25 U *Taq* and 0.25 U Vent DNA polymerase. PCR was performed in a thermal cycler from HYBAID (Model No. HB-TR1) and the reaction mix was overlaid with two droplets of mineral oil (Sigma). Cycling comprised 29 cycles of 60 s at 94°C, 30 s at 54°C, 60 s at 72°C and a single final elongation step of 120 s at 72°C. The PCR products were precipitated with ethanol and dissolved in 10 µl TE Buffer (10 mM Tris pH 7.5, 1 mM EDTA).

Plasmid pBSSK-hNAT2 (51) containing the wild-type human *NAT2* cDNA, i.e. the *NAT2**4 allele (52–54), was a generous gift of Prof. Dr U.Meyer (University of Basel, Switzerland). An *Eco*RI site was introduced 10 bp 5′ to the initiation codon of the *NAT2* gene in pBSSK-hNAT2 by site-directed mutagenesis using single-stranded DNA isolated from *E.coli* transformants upon infection with helper phage R408 (Stratagene, La Jolla, CA) and a 29 bp oligonucleotide (5′-TTTTATGTTTTG***GAATTC***TTAGGGG-3′, where altered bases are indicated in bold and the new *Eco*RI site in italic). The resulting 1.1 kb *Eco*RI fragment was subsequently isolated from an agarose gel and cloned in the *Eco*RI site of the centromeric yeast expression vector pHE46 (42). These manipulations resulted in plasmid pGP10, where the *NAT2* cDNA is flanked by the constitutive promoter of the yeast glyceraldehyde-phosphate-dehydrogenase gene (*GAPDH*) and the terminator of the yeast acid phosphatase gene (*PHO5*), respectively. In order to clone the *NAT2* expression cassette into a high copy vector, the 1.9 kb *Xho*I–*Sac*I fragment was first subcloned into the *Xho*I–*Sac*I-digested pCENEX645 (55) so that the *NAT2* expression cassette, now flanked by *Not*I sites, could be inserted into the unique *Not*I site of 2 µ vector pNW144 (56), resulting in vector pGP90 (Figure 1). To construct plasmid pGP100 the 5.7 kb *Xba*I fragment of plasmid pCS316 (43) containing the sequences for replication and selection in *E.coli*, the *URA3* gene for selection in yeast and part of the 2 µ sequence was isolated (Fig. 2). Self ligation of this fragment resulted in plasmid pMini harboring a single *Sph*I site between the *URA3* gene and the partial 2 µ sequence. The *Not*I fragment isolated from pGP90 was made blunt using Klenow polymerase

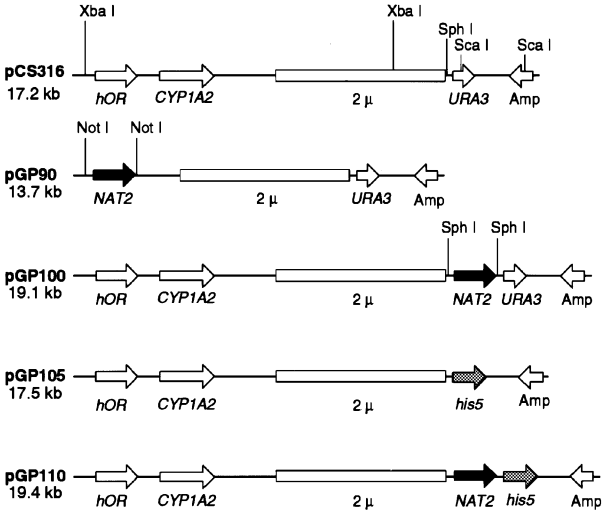

Fig. 1. Schematic representation of relevant yeast expression vectors. *CYP1A2*, *hOR* and selection markers are denoted as open arrows. The *hNAT2* expression cassette is depicted as black arrows and the *his5* marker as shaded arrows. The 2 µ sequences necessary for replication in yeast are depicted as open boxes. Only relevant restriction sites are shown and the map is drawn to scale.

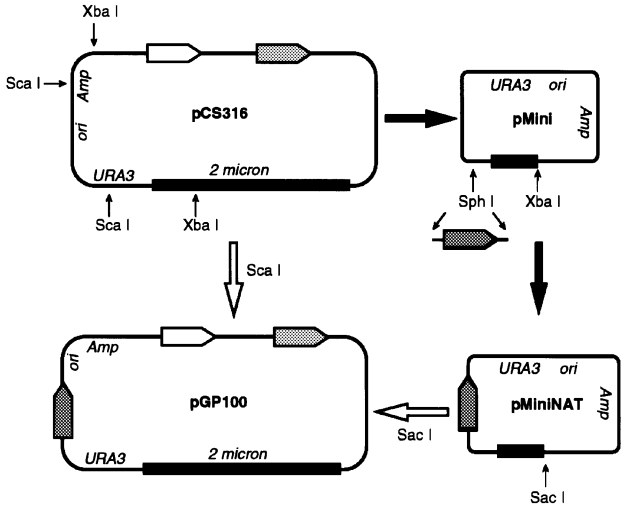

Fig. 2. Scheme describing the construction of vector pGP100. In a first step, plasmid pMini was constructed by selfligating the small *Xba*I fragment of pCS316. Subsequently, the *NAT2* expression cassette (black arrow) was inserted into the unique *Sph*I site of pMini to give pMiniNAT. The resulting plasmid was linearized at *Sac*I and cotransformed into yeast with the large *Sca*I fragment from pCS316 to give pGP100 upon *in vivo* recombination. Expression cassettes for human *CYP1A2* (open arrow) and *hOR* (shaded arrow) are depicted. The black bars represent 2µ sequences. The used restriction sites are indicated by small arrows. Plasmids are not drawn to scale.

and phosphorylated *Sph*I linkers (New England Biolabs) were added by ligase treatment. After heat inactivation of ligase, the fragment was first cleaved with *Sph*I and then, after purification by gel electrophoresis, ligated in the unique *Sph*I site of pMini, resulting in vector pMini-NAT.

pMini-NAT was linearized by *Sac*I digestion and isolated from an agarose gel. Plasmid pCS316 was digested with *Sac*I and the fragment containing the *CYP1A2* and *hOR* expression cassettes as well as the entire 2 µ sequence was isolated. Aliquots of 0.5 µg of both linear DNA fragments were co-transformed into yeast strain YS18 according to the protocol described by Klebe *et al*. (57). After selection of transformants on YM His, Leu plates, plasmids were rescued from yeast. For this purpose genomic yeast DNA was isolated by the method of Hoffmann and Winston (58) from 4 ml YM His, Leu cultures. After further phenol purification and ethanol precipitation 25–50% of the yeast DNA was used to transform chemically competent MC1061 according to the protocol described by Hanahan (59). The rescued plasmids were characterized by restriction analysis and the resulting plasmid pGP100 was transformed into yeast strains YB110 and YHE2. A crude description of these plasmid construction without any details has recently been presented in the form of a congress report (60).

Expression vectors pGP105 and pGP110 containing the *S.cerevisiae HIS3* homologue *his5* from *Schizosaccharomyces pombe* were constructed as follows: The *his5* gene of *S.pombe* located on vector pFA6a-HIS3MX6 (61) was amplified by PCR using primer S1 (5′-TAATCTCCGAACAGAAGGAAGAACGAAGGAAGGAGCACAGCGTACGCTGCAGGTCGAC-3′) and S2 (5′-CAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAATCGATGAATTCGAGCTCG-3′) exhibiting 18–19 nucleotides homology (underlined) to the pFA-HIS3MX6 multicloning site. The 5′ extensions of these primers contain 40 nucleotides which are homologous to the region immediately downstream of the start codon and to that upstream of the stop codon of the *S.cerevisiae URA3* gene on pCS316, respectively. Aliquots of 500 ng of the resulting 2 kb PCR product and 500 ng of undigested pCS316 or pGP100 were used to transform YS18. After 3 days selection on minimal plates containing Leu and Ura the colonies were streaked out on 5-FOA plates to eliminate clones that retained the *URA3* plasmids pCS316 or pGP100. Plasmids were rescued as described previously and transformed in KC8 *E.coli* cells. *Escherichia coli* transformants were first selected on LB Amp plates for 1 day at 37°C and then replicated onto *E.coli* M9 minimal plates containing 100 mg/l Leu, 20 mg/l Trp, 20 mg/l Ura and 100 mg/l ampicillin but lacking His to select for vectors which had replaced the *URA3* gene with the *his5* gene of *S.pombe*. Plasmids of such transformants were isolated and analyzed. The resulting vectors pGP105 (expressing human *CYP1A2* and *hOR*) and pGP110 (expressing human *CYP1A2*, *hOR* and *NAT2*) were used to transform the *his3* strain YGP1B.

Preparation of yeast protein extracts from S.cerevisiae cells

Protein extracts were prepared from cells growing exponentially at 30°C. Cells from a 100 ml culture were harvested by centrifugation and resuspended at 2×10^9 cells/ml in disruption buffer [0.1 M Tris pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) containing proteinase inhibitors (Complete Protease Inhibitor Cocktail; Boehringer Mannheim) after washing the cells twice in ice-cold disruption buffer. Subsequent to the addition of 2 vol chilled glass beads (0.45–0.55 mm), the cells were broken by heavy vortexing five times for 60 s. The efficiency of cell breakage was inspected under a microscope followed by recovery of the extract by use of a drawn out Pasteur pipette. The glass beads were washed once with 1 vol disruption buffer containing proteinase inhibitors. Cell debris was removed from the pooled extracts by a centrifugation step for 20 min at 15 000 *g* and the supernatant was recovered.

Immunological detection of human proteins in yeast extracts

For immunological detection of NAT2, supernatants from the yeast crude extracts described above were further purified by additional centrifugation at 100 000 *g* for 60 min at 4°C. The proteins in the supernatant were separated by SDS–PAGE as described by Laemmli (62) and transferred onto nitrocellulose membranes (Hybond ECL from Amersham). Blotting was performed in blotting buffer (25 mM Tris pH 8.3, 192 mM lycine, 20% MeOH) for 80 min at 100 V using the Mini Trans-Blot Transfer cell from Bio Rad (Glattbrugg, Switzerland). For immunological detection the membrane was first blocked overnight with 5% low-fat milk in TBST [5 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20 (v/v)] and then incubated for 3 h with a 1:1000 dilution of a rabbit polyclonal antiserum raised against human NAT (a generous gift from Dr D.Grant, Hospital for Sick Children, Ontario, Canada). To suppress non-specific binding of the rabbit serum, 0.8 ml protein extract (7.2 mg/ml) prepared from the parental strain YB110 was added to 10 ml of the diluted antiserum. After washing the membranes once for 15 min and twice for 5 min with TBST, a 1:3000 dilution of the secondary peroxidase-labelled antibody (purified goat anti-rabbit IgG; Bio-Rad) was added in 10 ml TBST, 5% (w/v) low-fat milk. After 1 h incubation with the secondary antibody, the membrane was washed with TBST as described before and peroxidase activity was detected using luminol as substrate (SuperSignal; Pierce, Rockford, IL) and exposing the blot to X-ray film (X-OMAT; Kodak, Rochester, NY) for 5–10 min.

Immunological detection of hOR and CYP1A2 in crude extracts was performed similarly, except that the blots were probed with a 1:1000 dilution of a rabbit antiserum directed against rat oxidoreductase commercially obtained from Oxygene (Dallas, TX), or a 1:1000 dilution of a rabbit antiserum against mouse CYP1A enzymes (a gift of Prof. U.A.Meyer).

Determination of NAT2 activity

Enzymatic activities of yeast extracts were measured essentially as described by Andres *et al*. (63). An aliquot of 50 µl yeast crude extract (5–10 mg protein/ml) was added to a final reaction-volume of 90 µl containing 0.1 mM SMZ and 0.5 mM acetylCoA. The reaction was stopped by adding 50 µl 25%

(w/v) TCA after an incubation for 30 min at 37°C, and 760 µl 5% (w/v) 4-dimethylamino-benzaldehyde dissolved in acetonitrile was added to detect unreacted substrate by measuring absorbance at 450 nm.

Recombination tests

The recombination tests with strain YHE2 or YB110 were carried out as described previously (64). In brief, exponentially growing cells in supplemented minimal medium (YM Ade, Ile, Trp, Leu, Val for YHE2 and YM Ade, His, Trp for YB110) were collected by centrifugation and resuspended in 0.1 M sodium phosphate buffer (pH 7.5) to a cell density of 10^7 cells/ml for strain YHE2, or 2×10^8 cells/ml for strain YB110, respectively. Aliquots of 1 ml of the cells were exposed to different concentrations of HAs for 4 h at 30°C in a rotary shaker. After exposure, the cells were collected by centrifugation, washed once and resuspended in supplemented minimal medium at a density of 5×10^6 for YHE2 or 8×10^8 cells/ml for YB110, respectively. Sample of 100 µl of the cells were plated directly on plates for the selection of Trp^+ or His^+ recombinants. To determine survivors the cells were appropriately diluted and spread on YPD plates. The plates were incubated at 30°C for 4 or 2 days, respectively.

Mutation assays

YGP1B transformants were grown in YM and exposed to mutagens at a density of 10^7 cells per ml in 0.1 M sodium phosphate buffer (pH 7.5). After 4 h the cells were washed with YPD and the survivors were titrated on YPD plates. To allow expression of the Ura^- phenotype, 0.2 ml of the cells was used to inoculate 10 ml YPD and incubated overnight in a rotary shaker at 30°C to allow growth for six to seven generations. The cells were resuspended to a density of 10^8 cells per ml and 100 µl aliquots were plated on YM His, Leu, Ura plates supplemented with 5-FOA for the selection of *ura3* mutants. To quantitate survivors, 100 µl aliquots of a 10^{-5} dilution were plated on YPD. Plates were incubated at 30°C and scored after 2 days for survivors and after 3 days for *ura3* mutants.

Statistical analysis

Recombination and mutation frequencies were analyzed statistically by performing a Student's *t*-test on the data.

Results

Extracellular activation of HAs may pose problems with yeast

In the standard Ames test (65), the inability of *S.typhimurium* cells to metabolize promutagens is solved by inclusion of rat liver S9 extract in the incubation mixture. Although this system works well with bacteria, we found that such extracellular metabolic activation of HA compounds was inefficient with yeast as the indicator organism. Compared with intracellular activation of HAs in yeast (see below), the incubation of HAs with S9 gave rise to only a marginal increase in translocation frequency, the genetic endpoint detected with strain YB110 (data not shown). This was not surprising taking into account the low acetylation activity detected with benzidine as substrate in S9 extract (66). For this reason we decided to express the necessary enzymes in our genotoxicity tester strains.

Construction of vectors for the expression of human NAT2 in S.cerevisiae

With the objective to obtain an *S.cerevisiae* strain able to metabolize HAs to the respective genotoxic products, a set of new vectors has been constructed for the expression of the wild-type human *NAT2* alone or in combination with human *CYP1A2* and *hOR* as described in Materials and methods and Figure 1. In brief, the *NAT2*4* cDNA was manipulated by site-directed mutagenesis and cloned between expression signals in a low copy yeast vector (Materials and methods). From there the expression cassette was recovered and inserted into vector pNW144 to give pGP90 which replicates at high copy number in yeast. Plasmid pGP100 for co-expression of human *NAT2*, *CYP1A2* and *hOR* was constructed by *in vivo* recombination because vector pCS316, which was the basis for pGP100, was considerably large (17.2 kb) and contained no single restriction sites for cloning of a further cassette. In brief the *NAT2* expression cassette was subcloned into pMini that contained partial homology to plasmid pCS316 (Figure 2). Linearized pMini and digested pCS316 were then co-transformed into the *ura3* strain YS18. Self-ligation of either plasmid fragment alone would not result in a functional replicating yeast vector because only the fragment from pMini contained the *URA3* gene for selection whereas only the pCS316 fragment contained the entire 2 µ sequence for the replication in yeast (Figure 2). Yeast transformants were plated on uracil-deficient medium and plasmids were rescued in *E.coli*. Subsequent restriction analysis showed that the majority (eight of nine) of the transformants contained the correct plasmid with all three expression cassettes, arguing that the *in vivo* recombination was very efficient.

The expression vectors described above carry the *URA3* gene as yeast selection marker. To further provide the possibility to use the genomic *URA3* gene for the identification of mutations, an additional set of expression vectors was constructed carrying the *his5* gene of *S.pombe* as selection marker. For this purpose the *his5* gene was first amplified in a PCR reaction using vector pFA6a-His3MX6 as template and the primers S1 and S2 (Materials and methods). The product of this PCR resulted in a complete *his5* gene flanked by 40 bp sequences that are homologous to nucleotides –149 to –110 upstream and 146–185 downstream of the *URA3* open reading frame on pCS316 or pGP100. Each circular vector and the PCR product were co-transformed in strain YS18 followed by rescue in *E.coli* as described in Materials and methods. Since the vectors contained no suitable restriction sites near or within the *URA3* gene, linearization was not possible, an experimental detail leading to a dramatic decrease in the formation of the desired recombination product. To overcome this problem and to eliminate non-recombinant plasmids, the His^+ transformants were further selected on 5-FOA-containing plates prior to rescue of the vectors in *E.coli* KC8. The *S.pombe his5* gene complemented the *hisD* mutation in KC8 allowing for selection of *his5*-containing transformants on M9 minimal plates (Materials and methods). Plasmid pGP105 and pGP110, which resulted from this selection procedure, were transformed in the *his3* strain YGP1B, upon a careful restriction analysis which revealed their correct structure (data not shown).

Immunological detection of human NAT2, CYP1A2 and hOR protein

To see whether the different human cDNAs were expressed to stable proteins in yeast harboring the newly constructed plasmids, crude extracts of such transformants were prepared. For the detection of NAT2 protein, the extracts were further purified by a high-spin centrifugation as described in Materials and methods, and NAT2 protein was detected in a western blot using a polyclonal rabbit antiserum raised against human NAT. As shown in Figure 3, the specific NAT2 band was only present in extracts prepared from pGP90 or pGP100 transformants, but not in extracts prepared from pNW144 or pCS316 carrying strains. The size of the detected band (31–32 kDa) was in good agreement with the calculated molecular mass (33.5 kDa) of human NAT2. The weak bands visible directly below the *NAT2* band are non-specific, since they appeared also in the control extracts prepared from pNW144 and pCS316 transformants. Nevertheless, the western blot confirmed that human NAT2 protein was successfully expressed in yeast into a stable protein.

CYP1A2 and hOR were also detected in the transformants,

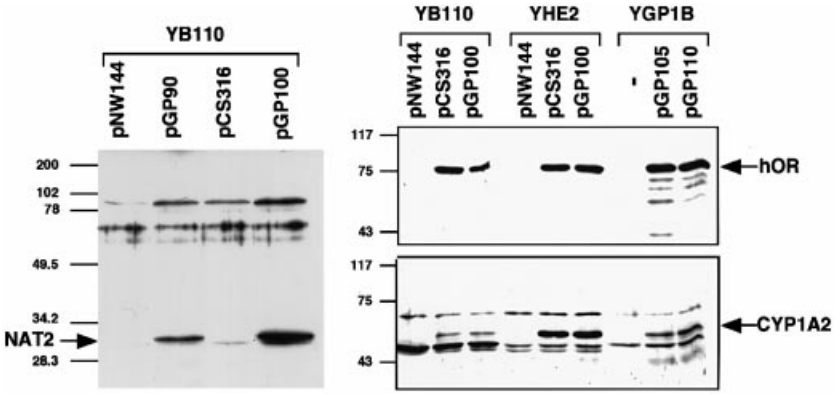

Fig. 3. Western blot analysis of human proteins expressed in yeast. High spin supernatants from crude extracts (100 μg protein/lane) of yeast strain YB110 transformed with the indicated plasmids (left blot) were electrophoresed on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose and immunoreacted with a polyclonal rabbit antiserum raised against human NAT. The upper and lower blots on the right derived from 7.5% SDS–polyacrylamide gels and each contained 50 μg protein per lane prepared from the indicated transformants. The blots were probed with an antiserum directed against rat NADPH-cytochrome P450 reductase (upper blot) or mouse CYP1A enzymes (lower blot). The arrows point to the specific bands on the blot. Size markers are indicated.

except in those transformed with vector pNW144 or in non-transformed YGP1B. In the case of hOR, clean specific bands were seen with some degradation products in strain YGP1B. Loading of equivalent amounts (50 μg protein per lane) was verified by staining the blots with Ponceau S (not shown) prior to probing with antisera, allowing a semi-quantitative evaluation of the expression level. In the case of CYP1A2, the antiserum detected for unknown reason some non-specific bands in addition to the indicated specific band. A comparison of band intensities argued for a somewhat higher *CYP1A2* expression level or protein stability in YHE2 transformants compared with YB110 and YGP1B transformants.

NAT2 activity in yeast

In order to test whether NAT2 activity was present in yeast, the same crude extracts that have been used for western blot analysis were subjected to a photospectrometric assay using SMZ as specific NAT2 substrate.

The results shown in Figure 4 clearly argued that human NAT2 was expressed in yeast to an enzymatically active form because only strains transformed with pGP90, pGP100 or pGP110 exhibited substantial NAT2 activity, whereas the strains transformed with either vector pNW144, pGP105 or plasmid pCS316 showed no detectable NAT2 activity. The fact that no NAT2-related activity was present in vector-transformed strains provided us with the opportunity to study the human enzyme in a clean background.

Successful expression of the *hOR* cDNA on plasmid pGP100 into functional enzyme was demonstrated by determination of cytochrome *c* reductase activity in crude extract (data not shown). Evidence for expression of *CYP1A2* into a functional enzyme was obtained indirectly (see below).

Activation of HAs to recombinogenic products in yeast

Having obtained evidence for faithful expression of the three human cDNAs into catalytically active enzymes in yeast, we investigated whether metabolically activated HAs would induce mitotic recombination in this organism. To address this question the *S.cerevisiae* strains YB110 and YHE2 transformed with pGP100 or other plasmids were exposed to HAs. Strain YHE2 allows monitoring of gene conversion between two defective *trp5* alleles which reconstitutes a functional *TRP5* gene, whereas strain YB110 detects chromosomal translocations between two truncated *his3* fragments located on non-homologous chromosomes resulting in a functional *HIS3* gene. Both recombination events provide an easily selectable phenotype and occur spontaneously at frequencies of $1.35 \pm 0.4 \times 10^{-5}$ and $1.7 \pm 0.9 \times 10^{-7}$, respectively, where means and standard deviations were determined from 28 values. These frequencies were well in line with previous data determined with the two strains (44,64), thus the expression of the human enzymes apparently had no influence on the occurrence of spontaneous recombination.

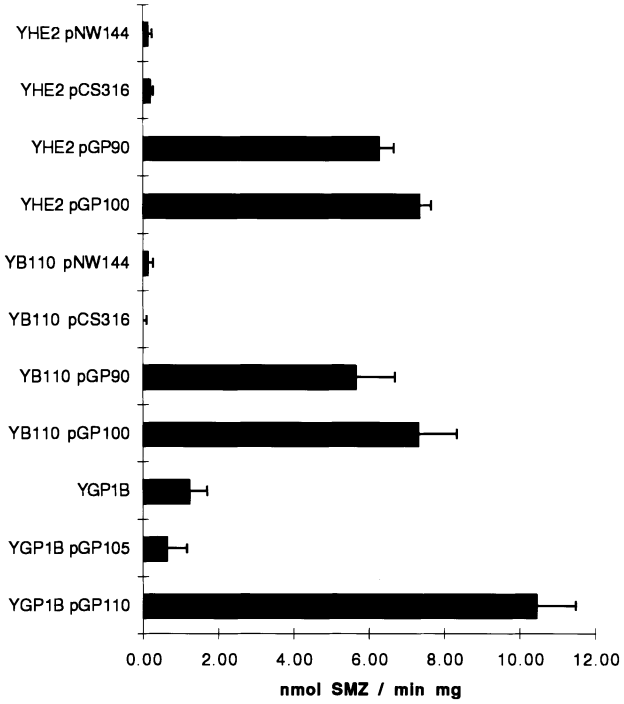

Fig. 4. Enzyme activity of the expressed NAT2 protein from crude extracts of yeast cells using SMZ as substrate. Fifty microliters of crude extracts, prepared from YHE2, YB110 or YGP1B transformants carrying the indicated plasmids were used for the assay. Values are represented as the means of three independent determinations. Standard deviations are represented by bars.

The yeast cells were exposed for 4 h to different concentrations of HAs and recombinants were scored as described in Materials and methods. When the cells were exposed to IQ,

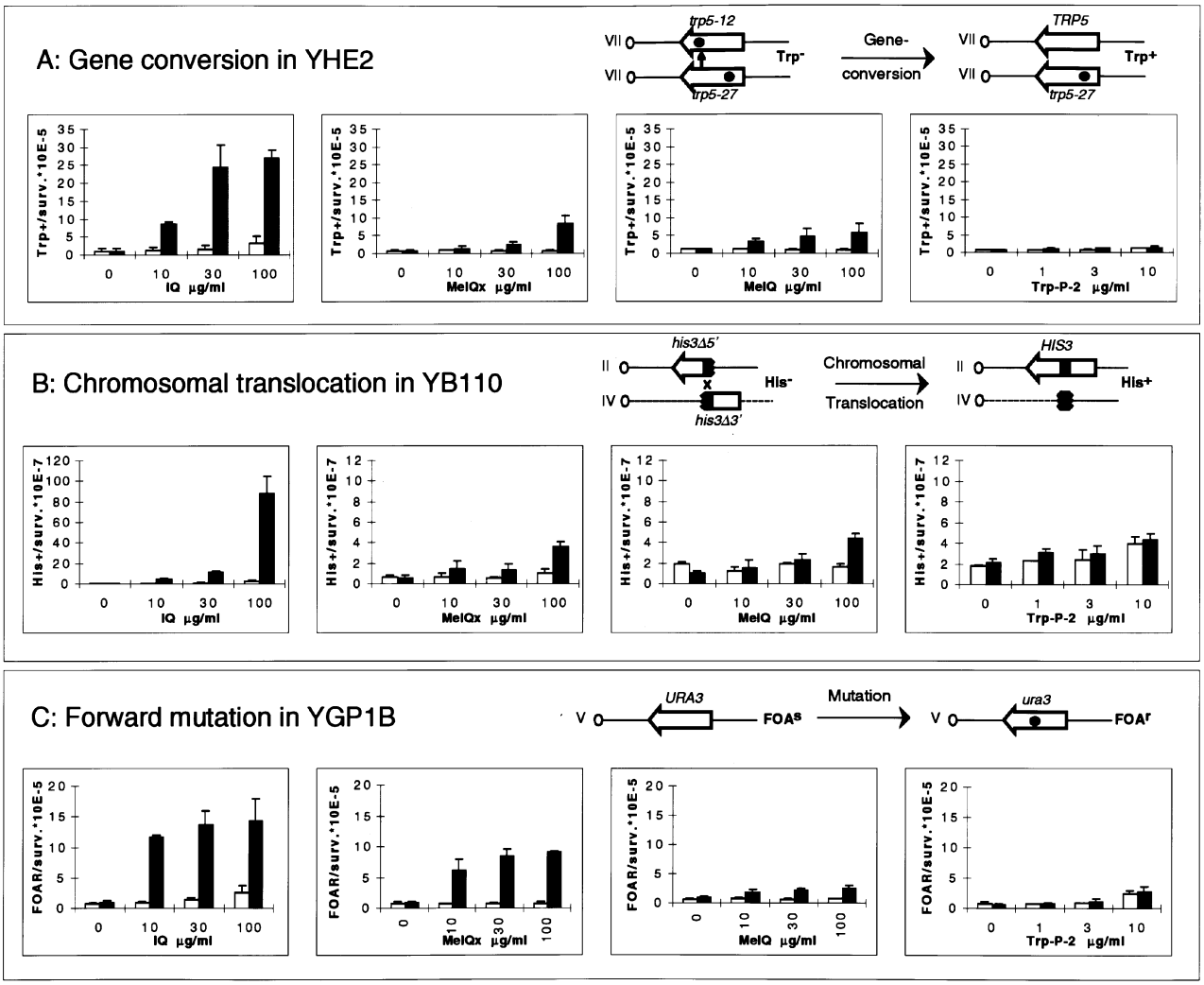

Fig. 5. HA-induced gene conversion, translocation and point mutation in yeast. The different genotoxic endpoints detected with the respective strains are depicted above each panel of graphs. Chromosomes are numbered with roman numerals. Mutations responsible for gene inactivation are depicted as filled circles. Strain YHE2 (**A**), YB110 (**B**) or YGP1B (**C**) expressing *CYP1A2* and *hOR* (white columns) or *CYP1A2*, *hOR* and *NAT2* (black columns) were exposed to HAs as described in Materials and methods. The means and standard deviations (bars) were determined from three independent experiments in all experiments except those with Trp-P-2 where only two experiments were performed. Note the different scale in (B) for the compound IQ.

dose-dependent increases in gene conversion and translocation frequencies were found. At the highest concentration tested this HA compound elevated the gene conversion frequency in strain YHE2 ~23-fold over the background and that of chromosomal translocation ~80-fold in strain YB110 (Figure 5A and B). In contrast both pCS316-transformed strains, which did not express human *NAT2*, showed no significant increase in mitotic recombination frequency, thus indicating that the activation of IQ to recombinogenic products was absolutely dependent on the presence of NAT2. Similarly, no induction of either recombination event was observed with the yeast strain carrying the parental vector pNW144, expressing neither *CYP1A2* nor *NAT2* or pGP90 expressing *NAT2* alone (data not shown). The HA compound 2-amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx) elevated the gene conversion and translocation frequency ~10- and 6-fold, respectively, over the background (Figure 5A and B). Again the genotoxic response was only seen in the presence of NAT2, whereas no or only an insignificant increase was detected in gene conversion and translocation frequencies, respectively, of pCS316 transformed strains.

Subsequent experiments were performed where the metabolically competent tester strains were exposed to additional HA compounds. These experiments revealed that 2-amino-3,4-dimethyl-imidazo-[4,5-f]quinoline (MeIQ) induced a maximal 4-fold increase in gene conversion and translocation, again only in the presence of CYP1A2 and NAT2. However, this increase was statistically not significant. With the other compounds, 2-amino-6-methyldipyrido-[1,2-a:3′,2′-d]imidazole (Glu-P-1), 2-aminodipyrido-[1,2-a:3′,2′-d]imidazole (Glu-P-2), 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) and 3-amino-1-methyl-5H-pyrido-[4,3-b]indole (Trp-P-2) the observed genotoxic response was too weak to be scored as positive. The weak albeit non-significant increase in recombination seen with PhIP (not shown) and Trp-P-2 (Figure 5A and B) appeared to be independent of *NAT2*. In all experiments, except those with Trp-P-2, the exposure of the tester strains to the different HA compounds was paralleled by only a weak cytotoxicity. At the highest dose applied, <20% of the cells were killed (data not shown). Since Trp-P-2 exerted a strong cytotoxic response, the highest dose was limited to 10 µg/ml.

Mutagenic activity of HAs in yeast

Since HA compounds are known as potent bacterial mutagens (10), we were interested to compare this activity to that detected in yeast. To this end forward mutations in the endogenous *URA3* gene were monitored by selection with 5-FOA. This compound is converted to a toxic product in *URA3* wild-type cells, whereas *ura3* mutants survive the selection. Mutations were studied in a haploid strain to ensure that a single *URA3* allele was present which could not be inactivated by a recombination-mediated process between homologues. Strain YGP1B was transformed with plasmid pGP110 expressing *CYP1A2*, *hOR* and *NAT2* from a vector carrying *his5* as selection marker. In parallel, YGP1B was transformed with control plasmid pGP105, lacking the *NAT2* expression cassette. Both strains were grown to exponential phase and exposed to IQ followed by selection for FOA^r mutants as described in Materials and methods. Mutation frequencies were expressed as FOA^r mutants/survivors.

A mean and standard deviation of $7.8 \pm 2.3 \times 10^{-6}$ for the frequency of spontaneous forward mutations in *URA3* were determined from 28 values. Upon exposure to IQ a 15-fold increase in the frequency of FOA^r mutants was observed (Figure 5C). The strain containing the control plasmid pGP105 showed a 3-fold increase in the mutation frequency. However, a similarly weak increase was seen in the untransformed strain (data not shown), suggesting the presence of a minor impurity in the compound used rather than a CYP1A2-independent activation by NAT2. A 9-fold NAT2-dependent increase in mutation frequency occurred upon exposure to MeIQx (Figure 5C), whereas Glu-P-1 gave rise to a 4.5-fold increase of the mutation frequency at 30 μg/ml (data not shown). MeIQ and Trp-P-2 induced only a small but non-significant increase in the mutation frequency, the latter even in the absence of NAT2 (Figure 5C) or the absence of either enzyme (data not shown). No response was seen with PhIP and Glu-P-2 (data not shown).

Discussion

This study describes a new experimental system, based on metabolically competent *S.cerevisiae* strains able to metabolize several HA compounds intracellularly, to study the genotoxic activity of HAs. Similar yeast strains expressing different human phase I enzymes have proven valuable tools to assess the genotoxic activity of environmental carcinogens (64,67). In this study we have concentrated on HAs, comparing their recombinogenic and mutagenic activity.

Since HA-induced recombinational events may be one of the causes that lead to LOH of cancer-related genes as well as to microsatellite instability observed in many tumors, we were interested to investigate their potential recombinogenic activity in yeast. Multiple indications for a possible recombinogenic activity of HAs are present in the literature. For example, Kitazawa *et al.* (68) identified PhIP-induced alterations of the two hypervariable minisatellites Pc-1 and Pc-2 in two mouse tumor cell lines by DNA fingerprinting, suggesting that PhIP induced recombinational mutations. Similarly, Toyota *et al.* (36) reported microsatellite instability (MI) and LOH in PhIP-induced rat mammary tumors. Examination of 62 microsatellite loci revealed MI in nine of 15 PhIP-induced mammary tumors. Five of these nine MI-positive tumors had mutations in more than one microsatellite locus. Moreover, four of these 15 PhIP-induced tumors revealed LOH of polymorphic loci located on chromosome 10 detected when different informative microsatellite markers were investigated, whereas no LOH was found in controls. Ushijima *et al.* (32) reported on genetic changes in the *p53* gene of MeIQ-induced forestomach tumors and tumor-derived cell lines of mice which were polymorphic at the *p53* locus. By use of single strand conformation polymorphism analysis and sequencing of PCR products, two of four tumors were found to carry single base substitutions in *p53* exons, one of which showed, in addition, LOH of the wild-type allele, whereas two tumors had no detectable alterations of *p53*. Analysis of tumor cell lines revealed that two of four had a base substitution and LOH, and the other two had double mutations (base substitutions and short deletions). Further analysis of the clones containing double mutations revealed that four types of alleles coexisted in these two cell lines which apparently resulted from recombination events within the *p53* gene. Oshima *et al.* (35) investigated the effects of PhIP on the intestinal polyp development in $Apc^{\Delta 716}$ mice heterozygous for a mutant *Apc* gene. At low doses of PhIP, a shift to larger sized polyps was observed and at higher doses, the number of polyps increased significantly. Interestingly, the increment was, in most cases, not paralleled by new mutations in the *Apc* gene, but by LOH leading to loss of the functional *Apc* allele, possibly by mitotic recombination.

All these observations prompted us to investigate the recombinogenic potential of HAs, and for this purpose new vectors have been constructed providing simultaneous expression of human *CYP1A2*, *hOR* and *NAT2* in yeast, an organism which allows us to quantify not only xenobiotic-induced mutations but also recombinations. Using this system we have found that several HAs were efficiently activated to products that induced both genetic endpoints, and unlike other systems, our tester organism potentially allows us to discriminate between HA-induced genotoxic insults that either lead to mutations or recombinations.

IQ exerted the strongest recombinogenic and mutagenic activity in our tester strains and the genotoxic response was absolutely dependent on the heterologously expressed enzymes. MeIQx was also efficiently activated by CYP1A2 and NAT2 and a clear induction of recombination and mutation has been observed. With several other HAs, i.e. MeIQ, Glu-P-1, Glu-P-2, Trp-P-2 and PhIP, weak but non-significant inductions of either genotoxic response were detected. The weak increases in PhIP and Trp-P-2 mediated mutation and recombination frequencies were NAT2 independent, a fact which is in accordance with data reported in earlier studies (69–71).

Below we will review other expression systems based on bacterial strains and mammalian cell lines that have been developed to study the genotoxic activity of HAs and compare them to the yeast system. The major aim of these experimental systems was to replace S9 as an activating system, since the enzymatic activities of S9 extracts are difficult to control or, in the case of certain phase II reactions, not sufficient. Additionally, by expressing human enzymes the problem of species specificity is solved and the metabolism inside the cell guarantees that the reactive metabolites are produced in proximity to the genetic target.

Josephy *et al.* (72) constructed a new *S.typhimurium* strain co-expressing human *CYP1A2* and *Salmonella O*-acetyltransferase (OAT). Although several aromatic amines were moderately activated intracellularly to reactive mutagens, detected by reversion of the *hisD3052* allele, extracellular activation by S9 extract was much more effective. The reason for this

considerably higher activity was probably the lack of NADPH-cytochrome P450 oxidoreductase in their heterologous expression system, and the problem was recently solved by Suzuki *et al.* (73) who constructed the *S.typhimurium* strain TA1538/ARO expressing human *CYP1A2*, *hOR* and *Salmonella* OAT. This new strain was highly susceptible to several HAs. The strongest response was observed with MeIQ, an observation which differs from ours where IQ gave the strongest response. The authors also demonstrated the implication of intracellular metabolism: the response of strain TA1538/ARO towards IQ was considerably higher than that of parental strain TA1535 exposed to IQ that was preincubated with a combination of the cytosolic and microsomal fractions prepared from TA1538/ARO. Recently, Josephy *et al.* (74) also described the construction of an *E.coli* strain able to bioactivate HA compounds. Strain DJ4309 expresses *Salmonella* OAT from one plasmid and human *CYP1A2* and *hOR* from a second plasmid, and it carries a mutant *lacZ* gene on an F′ episome which reverts to *lacZ*$^+$ by a specific frameshift mutation. However, despite the presence of hOR in the cells, a 10 times stronger mutagenic response occurred, when IQ or MeIQ were activated extracellularly by S9 mix and it was argued that other CYP enzymes present in S9 and different from CYP1A2 might also be involved in the metabolism of the two HA compounds. In contrast, a stronger mutagenic response was detected when the arylamine compound 2-aminoanthracene was metabolized intracellularly, a discrepancy which remained elusive. After intracellular activation of IQ and MeIQ by CYP1A2, hOR and OAT, a 5-fold increase of *lacZ* revertants was found. The fact that no clear response was seen with the same compounds in the strain expressing *CYP1A2* and *NAT2* alone underlined the importance of P450 reductase as electron donor in *E.coli*. The IQ-induced increases of *lacZ* revertants found by Josephy *et al.* are comparable with our results in yeast with the minor difference that MeIQ gave only rise to a marginal increase in the mutation frequency detected in our system. Nevertheless, compared with the *Salmonella* system, the responses of HAs in *E.coli* and yeast are weak. An analysis of the mutation data determined with the *Salmonella* system (8,17,73,75) revealed MeIQ as the most potent mutagenic HA compound, followed by IQ and MeIQx which are ~5–10-fold less mutagenic.

Thompson *et al.* (75) compared the mutagenic potencies of S9 activated HAs detected with the Ames/*Salmonella* reversion assay with those detected at the *hprt* locus in nucleotide repair deficient Chinese hamster ovary cells (CHO). The mutagenic potency of MeIQ in the CHO-UV5 cell line was very weak compared with that found in *Salmonella*. PhIP produced the strongest responses concerning *hprt* mutation induction, cytotoxicity and sister chromatid exchanges, whereas this compound is weakly mutagenic in the Ames assay. None of the IQ-type compounds, which are potent *Salmonella* mutagens, gave a strong response. The reason for this was presumably the lack of NAT activity in CHO cells.

This problem was recently solved by Thompson *et al.* (76) by introducing the cDNAs for the expression of mouse *CYP1A2*, human *NAT2* or *Salmonella* OAT in the CHO-UV5 cell line. Exposure to IQ gave rise to a 1000-fold increase in cytotoxicity and *hprt* mutation induction compared with cell lines expressing exclusively *CYP1A2*. In NAT-expressing cell lines, IQ was 10-fold more potent than PhIP, a result which is well in line with ours in yeast. The authors also reported that intracellular activation of PhIP resulted in a 10-fold elevated sensitivity concerning cytotoxicity and mutation induction compared with S9-activated PhIP in CHO-UV5 cells. The results were confirmed by Wu *et al.* (77) by determining IQ and PhIP induced cytotoxicity, *aprt* mutations, chromosomal aberrations and sister chromatid exchanges in the same cell line. IQ induced the strongest responses in metabolically competent CHO cells as well as in our competent yeast cells, followed by MeIQx. Apart from PhIP, little is known about the genotoxic activity of other HA compounds in CHO cells expressing NAT. This question is of special interest since PhIP and MeIQx are by far the most abundant HA products present in cooked meats (1,8,78) and are thus more relevant with respect to human exposure.

Yanagawa *et al.* (79) examined the genotoxic effects of MeIQx and IQ in Chinese hamster lung (CHL) cells expressing human *CYP1A2*, *NAT1* or *NAT2* and guinea pig NADPH-cytochrome P450 oxidoreductase. A 370- and 100-fold increase in the cytotoxicity of IQ and MeIQx, respectively, was detected in the CHL cells expressing *CYP1A2*, *OR* and *NAT2* compared with the parental cells. Moreover, in the metabolically competent cell line the mutagenicity of IQ was 2.5-fold higher than that of MeIQx, an observation which is very similar to ours with yeast.

These and the other similarities with the Chinese hamster cell system let us conclude that the yeast system better reflects the mammalian than the bacterial system. Nevertheless, major differences exist between the mammalian and yeast systems. First of all the concentrations required to cause significant genotoxic response in yeast were ~100–1000-fold higher than in CHO or CHL cells and several reasons may exist for this: (i) our strains do not harbor any mutations in DNA repair genes as does the CHO-UV5 cell lines. Thompson *et al.* (75,76,80) have demonstrated that introduction of a mutant NER gene resulted in a 15-fold increase in HA-sensitivity. (ii) The yeast cell wall may pose a problem in the uptake of exogenous compounds and the low cytotoxicity (10–20% cell killing) seen upon HA exposure supports this assumption. (iii) Other processes including detoxification reactions may modulate the genotoxic reactions.

All these new cell systems constitute sensitive tools for assessing the genotoxicity of compounds requiring metabolic activation as well as for studying the molecular processes by which DNA damage can lead to mutation and cancer. Nevertheless, little has been known about the potency of HA to induce mitotic recombination in eukaryotic cells. Here we have shown that the recombinogenic activity of HAs is at least as pronounced as the mutagenic activity. Thus, as has been discussed recently for the mycotoxin aflatoxin B1 (38,64), the combination of mutagenic and recombinogenic activity may be responsible for the carcinogenic activity of HA compounds.

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