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CYTOCHROME P-450-MEDIATED ACTIVATION OF PROCARCINOGENS AND PROMUTAGENS TO DNA-DAMAGING PRODUCTS BY MEASURING EXPRESSION OF *umu* GENE IN *SALMONELLA TYPHIMURIUM* TA1535/pSK1002

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Abstract—A simple and sensitive procedure for the determination of cytochrome P-450 (P-450)-mediated activation of chemical procarcinogens and promutagens to DNA-damaging products has been developed using a method measuring the expression of the *umu* gene in *Salmonella typhimurium* TA1535/pSK1002, which is based upon the initial procedures as described by Oda *et al.* [*Mutation Res.* **147**, 219 (1985)]. The chemicals examined were a variety of potent carcinogenic and mutagenic compounds including heterocyclic aromatic amines, aromatic amines, polycyclic aromatic hydrocarbons and aflatoxin B_1. These chemicals were incubated with rat liver microsomes or a reconstituted monooxygenase system containing three forms of purified P-450 in the presence of a bacterial tester strain, and the induced-*umu* gene expression was determined by measuring the β-galactosidase activity produced by fusion gene in the cells. The activity was increased linearly for at least 2 hr with an initial lag time of 30 min and was dependent on the concentrations of P-450 in the reaction mixture. Thus, the metabolic activation of these compounds by P-450 could be compared on a basis of the specific β-galactosidase activity/min/nmol P-450. Among three forms of P-450, two isozymes induced by 3-methylcholanthrene were found to be more active in catalyzing the metabolic activation of most of the chemicals examined than a form of P-450 which is induced by phenobarbital. Data also showed that a high spin form of P-450 isolated from 3-methylcholanthrene-treated rats had a profound role in the activation of procarcinogens and promutagens. This conclusion was based on the results of catalytic activities by three forms of P-450 in a reconstituted monooxygenase system, and on the effects of specific antibodies against these P-450s on the reactions catalyzed by liver microsomes.

Very recently, a new test system, namely the *umu* test, for screening potential DNA-damaging agents was developed by Oda *et al.* [1], who showed that the results of this test are in agreement with the results of a well-known mutagenicity assay reported by Ames *et al.* [2, 3] and may be more useful with respect to simplicity, sensitivity, and rapidity. The *umu* test system is based upon the abilities of carcinogens and mutagens to induce expression of an *umu* gene in *Salmonella typhimurium* TA1535/pSK1002† in which a plasmid pSK1002 carrying a fused gene *umuC'-'lacZ* had been introduced; the *umu* gene seems to be involved in mutagenesis more directly than other known-SOS genes [4, 5]. The usefulness in detecting procarcinogens and promutagens after their metabolic conversions to reactive electrophiles by liver 9000 *g* supernatant fraction has also been reported in the *umu* test system [1]. To compare the abilities of a wide variety of environmental chemicals to induce *umu* gene expression, however, more detailed experiments employing kinetic analysis and the possible role of activating enzymes such as multiple forms of P-450‡ are required.

In this paper, we show that the expression of the *umu* gene in *S. typhimurium* TA1535/pSK1002, as determined by β-galactosidase activity, was increased by fifteen procarcinogens and promutagens when incubated with rat liver microsomes or a reconstituted monooxygenase system containing three forms of P-450. The chemicals used in this study were a variety of potent carcinogenic and mutagenic

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† Abbreviations: *S. typhimurium*, *Salmonella typhimurium*; PB, phenobarbital; MC, 3-methylcholanthrene; KC-500, a polychlorinated bipheny mixture containing 55% chlorine; P-450, liver microsomal cytochrome P-450; PB-1, a major form of P-450 induced by PB; MC-1, a low spin form of P-450 induced by MC; MC-2, a high spin form of P-450 induced by MC; IgG, immunoglobin G; 2,4,2',5'-TCB, 2,4,2',5-tetrachlorobiphenyl; 3,4,3',4'-TCB, 3,4,3',4'-tetrachlorobiphenyl; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-b]indole; Glu-P-1, 2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole; IQ, 2-amino-3-methyl-imidazo[4,5-f]quinoline; MeIQ, 2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline; 2-AA, 2-aminoanthracene; AFB_1, aflatoxin B_1; B[*a*]P, benzo[*a*]pyrene; 1,2,3,4-DBA, 1,2,3,4-dibenzanthracene; 2-AAF, 2-acetylaminofluorene; 7,12-DMBA, 7,12-dimethylbenzanthracene; AαC, 2-amino-9*H*-pyrido[2,3-b]indole; and MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-b]indole.

‡ The nomenclature used in this study to designate individual forms of P-450 has been described by Kuwahara *et al.* [6] in comparison to equivalent or corresponding forms characterized by other investigators.

compounds including heterocyclic aromatic amines, aromatic amines, polycyclic aromatic hydrocarbons and AFB_1. The increase in expression was dependent on an incubation time of between 30 and 90 min with an initial lag time of 30 min and on the concentrations of P-450 in the reaction mixture. Thus, the abilities of various forms of P-450 to activate these compounds in the *umu* test system were ascertained on the basis of specific β-galactosidase activity/min/nmol P-450, and the substrate specificities of three forms of P-450 were suggested. Data are also presented that a high spin form of P-450 isolated from MC-treated rats has a profound role for most of the chemicals examined. This conclusion was based on the results of catalytic activities by three forms of P-450 in a reconstituted monooxygenase system and on the effects of specific antibodies against these P-450s on the activities of liver microsomes.

MATERIALS AND METHODS

Materials. The chemicals used were obtained from the following sources: Trp-P-1 and Trp-P-2 from the Wako Pure Chemical Co. (Osaka); MC and PB from the Katayama Chemical Co. (Osaka); AFB_1 from the Makor Chemical Co. (Israel); 2-AAF, 2-AA, B[*a*]P, 1,2,3,4-DBA, and 7,12-DMBA from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); furylfuramide from the Ueno Pharmaceutical Co. (Osaka); polychlorinated biphenyl mixture KC-500 from the Kanegafuchi Kagaku Co. (Osaka); 2,4,2′,5′-[^{14}C]TCB from the Tokai Establishment of the Japan Atomic Energy Institute; and 3,4,3′,4′-[^{14}C]TCB from the New England Nuclear Co. (Boston, MA, U.S.A.). Glu-P-1, IQ, MeIQ and MeIQx were gifts from Dr. M. Nagao, and AαC and MeAαC from Dr. S. Sato of the National Cancer Center Research Institute (Tokyo). $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from the Oriental Yeast Co. (Osaka). The enzyme substrate *o*-nitrophenyl-β-D-galactopyranoside for the determination of β-galactosidase activity was obtained from the Wako Pure Chemical Co. All the other chemicals and reagents were standard products of analytical grade and were used without further purification.

Treatment of animals and preparation of microsomes. Male Sprague–Dawley rats, weighing about 150 g, were pretreated with PB dissolved in saline, MC dissolved in olive oil, or KC-500 dissolved in olive oil, by injecting i.p. for three consecutive doses; the daily dose was 80 mg/kg body weight for PB, 50 mg/kg body weight for MC, or 100 mg/kg body weight for KC-500. The animals were killed after being starved for 24 hr following the last injection. Liver microsomes were prepared as described previously [7].

Purification of microsomal enzymes. A form of P-450, designated PB-1, was purified from liver microsomes of PB-pretreated rats by the method of Harada and Omura [8]. This form is the main P-450 species in PB-induced rats and had a specific content of 16.6 nmol/mg protein. Another two forms of P-450, termed MC-1 and MC-2, were purified from liver microsomes of MC-pretreated rats by the methods of Kuwahara *et al.* [6] and were shown to be low and high spin types, respectively, of P-450. The specific contents of MC-1 and MC-2 were 18.0 and 15.4 nmol/mg protein respectively. All of the purified preparations of P-450 exhibited a single protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A preparation of NADPH–P-450 reductase that was homogenous by gel electrophoresis was purified from liver microsomes of PB-pretreated rats by the method of Yasukochi and Masters [9] as modified by Taniguchi *et al.* [10]; its NADPH–cytochrome *c* reductase activity was 40 units/mg protein as determined by the method of Imai [11].

Rabbit antisera against purified PB-1, MC-1, and MC-2 were obtained by a method described previously [12, 13]. The IgGs were prepared by treatment of the antisera with ammonium sulfate fractionation followed by purification with DEAE-cellulose colum chromatography as described by Kaminsky *et al.* [12].

The bacterial strain. The bacterial tester strain *S. typhimurium* TA1535/pSK1002, containing a plasmid pSK1002 carrying a *umuC′-′lacZ* fused gene that produces a hybrid protein with β-galactosidase activity and whose expression is controlled by the *umu* regulatory region, was prepared as described previously [1, 5]. The bacteria were grown in either Luria broth [14] or TGA medium (1% Bacto tryptone, 0.5% NaCl and 0.2% glucose) supplemented with 20 μg/ml ampicillin. The overnight culture of the tester bacterial strain was diluted 50-fold with TGA medium and was further cultured until the bacterial density reached an absorbance at 600 nm of 0.25 to 0.3.

Analytical and assay methods. The activation of *umu* gene expression by activated procarcinogens and promutagens was determined as follows. The standard reaction mixture (final volume of 1.0 ml) contained: 50 mM potassium phosphate buffer (pH 7.25); a 0.01 to 0.08 mM concentration of one of the procarcinogens or promutagens, or a directly acting mutagen, furylfuramide, dissolved in 10 μl of dimethyl sulfoxide; 0.1 mM EDTA; an NADPH-generating system consisting of 0.25 mM $NADP^+$, 5 mM glucose-6-phosphate, 0.5 units of glucose-6-phosphate dehydrogenase and 1.5 mM $MgCl_2$; liver microsomes (containing about 0.03 nmol of P-450) or a reconstituted monooxygenase system containing 0.01 nmol of PB-1, MC-1 or MC-2, 0.1 unit of NADPH–P-450 reductase, 5 μg of dilauroylphosphatidylcholine and 0.1 mg of sodium cholate; and 0.75 ml of bacterial solution described above. The reaction was allowed to run for 30, 60 and 90 min at 37° and then was terminated by rapid cooling in an ice-water bath. A 0.2-ml portion of the resulting mixture was used for the assay of β-galactosidase activity according to the method of Miller [14] as described by Oda *et al.* [1], and the bacterial density was measured at 600 nm for the remaining portion of 0.8 ml. The unit of enzyme activity was calculated by the method of Miller [14]. The *umu* gene was expressed as specific β-galactosidase activity (unit)/min/ml or nmol P-450.

The cytochrome P-450-dependent activations of 2,4,2′,5′-[^{14}C]TCB and 3,4,3′,4′-[^{14}C]TCB to protein-bound metabolites, which have been reported in

this laboratory to be good measures of the substrate specificities of PB-1 and MC-1 respectively [15], were determined as follows. The reaction mixture (final volume, 1.0 ml) contained the same components as in the *umu* test, except that the bacterial solution was omitted. The mixture was incubated at 37° for 15 min with 6 μM 2,4,2′,5′-[^{14}C]TCB or 10 μM 3,4,3′,4′-[^{14}C]TCB, and the metabolites bound to microsomal proteins were determined as described previously [15].

Cytochrome P-450 and protein contents were determined by the methods of Omura and Sato [16] and Lowry *et al.* [17] respectively. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [18].

RESULTS

Relation between incubation time and the activation of umu *gene expression by furylfuramide and Trp-P-2.* Our first experiments compared induction of *umu* gene expression by two chemicals, a directly acting mutagen, furylfuramide, and a promutagen, Trp-P-2, according to the procedures described in Materials and Methods. In the case of Trp-P-2, liver microsomes from KC-500-pretreated rats fortified with an NADPH-generating system were included in the reaction mixture to activate this promutagen to its intermediate metabolites which were further metabolized to ultimate mutagenic forms by an *N*-hydroxyarylamine *O*-acetyltransferase in the bacteria [19, 20]. Figure 1 shows the dependence of *umu* gene expression by furylfuramide on incubation time; the figure also compares the bacterial growth rate in the presence or absence of mutagen. To improve the initial linearity of the activation of *umu* gene expression, the effects of preincubation times of 0, 10, and 30 min, before adding furylfuramide, were compared. The bacterial growth rate appeared to be retarded by furylfuramide, with or without preincubation (Fig. 1A). Figure 1B shows that the furylfuramide-induced activation of *umu* gene expression increased linearly between 30 and 120 min of incubation time, whereas the initial reaction lagged in the first 30 min with or without preincubation time. In the absence of furylfuramide, *umu* gene expression did not increase with time; the basal level was about 100 units of β-galactosidase activity/0.2 ml. The linear increases in gene expression by furylfuramide between 30 and 90 min were almost the same for the different preincubations.

Figure 2 shows the kinetic analysis of the activation of *umu* gene expression by Trp-P-2 after its aerobic oxidation by liver microsomes from KC-500-pretreated rats in the presence or absence of an NADPH-generating system. As in the case for furylfuramide, the bacterial cell growth rate also appeared to be retarded somewhat by Trp-P-2 after metabolic activation by liver microsomes. Moreover, a linear increase in activation of *umu* gene expression occurred between 30 and 120 min of incubation with liver microsomes, irrespective of uses of preincubation, although the reaction lagged for 30 min to a somewhat greater extent than that observed with a directly acting mutagen, furylfuramide.

Dependence of activation of umu *gene expression by liver microsomes on P-450 and substrate concentrations.* From these results, the following experiments were done by measuring *umu* gene expression using incubation times of 0, 30, 60 and 90 min without preincubation, and specific *umu* gene expression was calculated from the results of incubation between 30 and 90 min. The unit of β-galactosidase activity was calculated by subtracting the basal level from the experimental values.

Figure 3A shows that the activation of *umu* gene expression by Trp-P-2 or AFB_1 increased linearly with the concentration of P-450 up to 0.06 μM in the system containing liver microsomes from KC-500-treated rats. The maximum activations were attained in P-450 concentrations of 0.06 to 0.09 μM for Trp-P-2 and 0.09 to 0.12 μM for AFB_1. Figure 3B shows

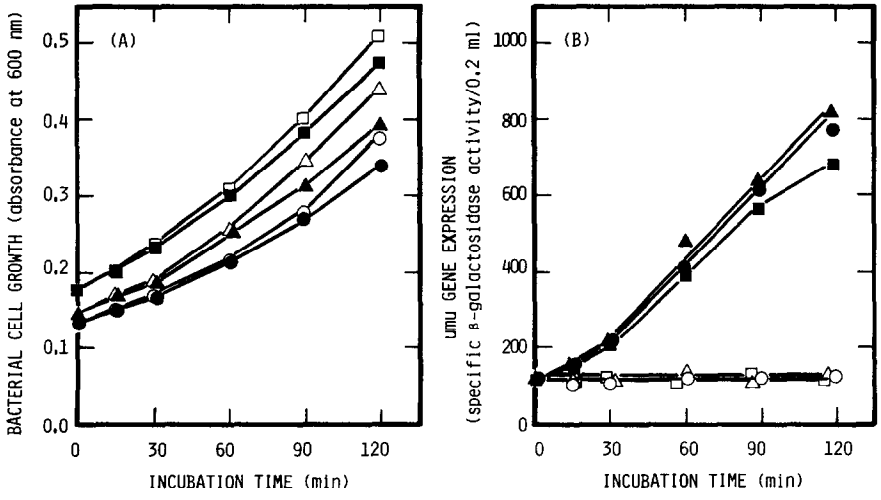

Fig. 1. Relation between preincubation time and bacterial growth rate (A), and activation of *umu* gene expression (B) by furylfuramide. The bacterial tester strain was preincubated at 37° for 0 min (○, ●), 10 min (△, ▲) or 30 min (□, ■), and then was incubated with (●, ▲, ■) or without (○, △, □) 0.01 mM furylfuramide. The bacterial cell number was expressed as a measurement of density at 600 nm.

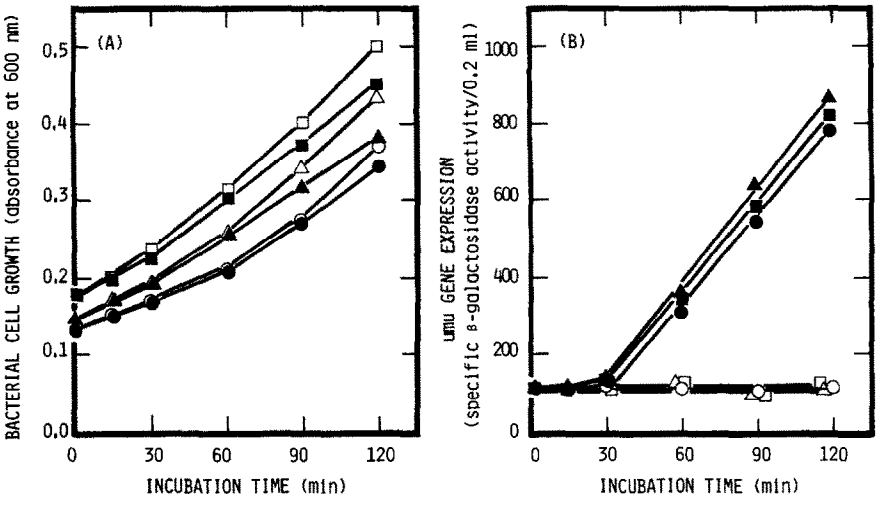

Fig. 2. Relation between preincubation time and bacterial growth rate (A), and activation of *umu* gene expression (B) by Trp-P-2 in rat liver microsomes. The bacterial tester strain was preincubated at 37° with 0.01 mM Trp-P-2 and liver microsomes from KC-500-pretreated rats for 0 min (○, ●), 10 min (△, ▲) or 30 min (□, ■), and then was incubated with (closed symbols) or without (open symbols) an NADPH-generating system for indicated periods of time.

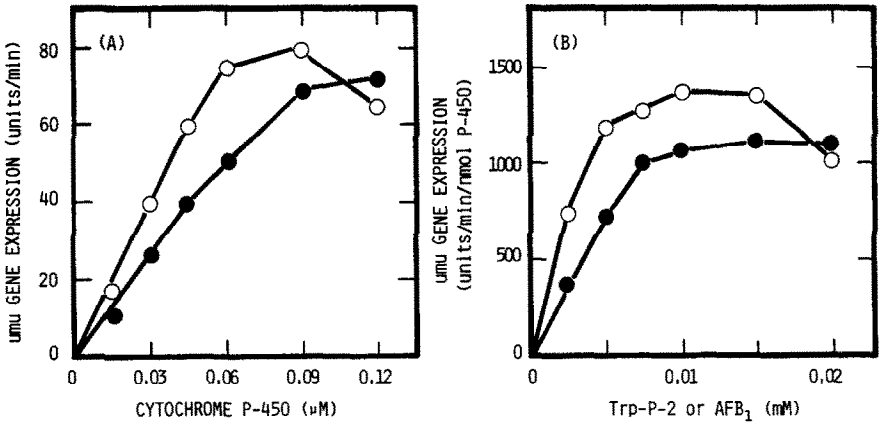

Fig. 3. Dependence of the activation of *umu* gene expression on the concentration of liver microsomal P-450 (A) and of substrates (B). The bacterial tester strain was incubated with liver microsomes from KC-500-pretreated rats in the presence of Trp-P-2 (○) or AFB_1 (●), and the activated *umu* gene expression was determined as described in Materials and Methods. The substrate concentrations for panel A were 0.01 mM, and the P-450 concentration for panel B was 0.03 μM.

Table 1. Effects of PB, MC, and KC-500 on liver microsomal cytochrome P-450 content and monooxygenase activities in the rat

	Cytochrome P-450 (nmol/mg protein)	TCB-binding activity (pmol/min/nmol P-450)	
		2,4,2′,5′-TCB	3,4,3′,4′-TCB
Control	1.13 ± 0.15	8.47 ± 2.30	1.40 ± 0.27
PB	2.52 ± 0.26*	21.4 ± 4.60*	0.40 ± 0.13*
MC	2.74 ± 0.19*	1.45 ± 0.23*	13.4 ± 1.48*
KC-500	2.80 ± 0.35*	14.2 ± 1.65*	5.77 ± 1.02*

Treatment of animals with inducers and determination of liver microsomal cytochrome P-450 content and monooxygenase activities were performed as described in Materials and Methods. Each value represents the mean of four animals ± SD.

* $P < 0.05$, as compared with the activities from untreated rats.

Table 2. Activation of *umu* gene expression by procarcinogens and promutagens in liver microsomes from untreated and PB-, MC- and KC-500-treated rats

	Conc (mM)	Activation of *umu* gene expression (units/min/nmol P-450)			
		Untreated	PB-treated	MC-treated	KC-500-treated
Trp-P-1	0.01	290 ± 30	100 ± 10*	790 ± 100*	420 ± 30*
Trp-P-2	0.01	160 ± 20	30 ± 10*	760 ± 80*	390 ± 40*
Glu-P-1	0.01	100 ± 20	30 ± 10*	810 ± 70*	390 ± 30*
IQ	0.01	500 ± 80	210 ± 20*	1200 ± 110*	450 ± 40
MeIQ	0.01	1000 ± 120	550 ± 60*	600 ± 40*	430 ± 40*
MeIQx	0.01	190 ± 10	50 ± 20*	820 ± 30*	620 ± 50*
2-AA	0.01	480 ± 40	140 ± 10*	790 ± 80*	560 ± 50
AFB_1	0.01	1300 ± 120	790 ± 80*	1010 ± 120	850 ± 90*
B[*a*]P	0.01	50 ± 10	60 ± 10	190 ± 20*	90 ± 20
1,2,3,4-DBA	0.01	30 ± 10	30 ± 10	140 ± 20*	50 ± 10
2-AAF	0.01	40 ± 10	40 ± 20	50 ± 20	40 ± 10
7,12-DMBA	0.01	40 ± 10	40 ± 20	140 ± 30*	70 ± 20
MC	0.01	<10	<10	90 ± 10*	30 ± 10*
AαC	0.08	<10	<10	530 ± 70*	110 ± 20*
MeAαC	0.08	160 ± 10	<10	540 ± 40*	410 ± 50*

Each value represents the mean of duplicate determinations ±SD.
* $P < 0.05$, as compared with the activities of liver microsomes from untreated rats.

that the substrate concentration in the reaction mixture was saturated by 0.005 to 0.015 mM for Trp-P-2 and 0.0075 to 0.015 mM for AFB_1. We also determined the activation of *umu* gene expression by various concentrations of other procarcinogens and promutagens in the reaction mixture and found that 0.01 mM was optimal for the assay of most of the chemicals examined except for AαC and MeAαC in which 0.08 mM substrate concentration was required. On the basis of these results, the following experiments were carried out using 0.01 to 0.06 μM P-450 and a 0.01 mM concentration of each of the substrates examined except for AαC and MeAαC.

Metabolic activation of procarcinogens and promutagens by liver microsomes. To address the possible role of individual forms of P-450 in the metabolic activation of procarcinogens and promutagens, the effects of PB, MC, and KC-500 on the catalytic activities by liver microsomes were examined in the *umu* test system. Table 1 compares the P-450 contents and the monooxygenase activities of liver microsomes used in this study. Three inducers caused marked increases in cytochrome P-450 content of 126, 142 and 148% for PB, MC, and KC-500 respectively. The conversion of 2,4,2',5'-TCB to protein-bound metabolites was catalyzed most actively by liver microsomes from PB-treated rats and was least active in liver microsomes from MC-treated rats. Conversely, MC treatment caused a marked enhancement of 3,4,3',4'-TCB binding activity, whereas PB did not. KC-500 gave an intermediate effect between PB and MC in both reactions. Thus, the substrate specificities of these microsomes were as reported previously [15].

Table 2 presents the results of activation of *umu* gene expression by procarcinogens and promutagens in the system containing these liver microsomes from untreated, and PB-, MC-, and KC-500-treated rats. Induction with PB caused little effect on microsome-catalyzed activation of the various chemicals examined as compared with the results from untreated rats. Particularly, the metabolic activations of some procarcinogens and promutagens including Trp-P-1, Trp-P-2, Glu-P-1, IQ, MeIQ, MeIQx, 2-AA, and MeAαC were lowered by PB on the basis of P-450 content. MC treatment, on the other hand, gave markedly increased activation of *umu* gene expression by procarcinogens and promutagens. These compounds were Trp-P-1, Trp-P2, Glu-P-1, IQ, MeIQx, 2-AA, B[*a*]P, 1,2,3,4-DBA, 7,12-DMBA, MC, AαC and MeAαC. The activations of AFB_1 and MeIQ by liver microsomes from untreated rats were much higher than those from PB- and MC-treated ones. KC-500 also caused an induction of metabolic activation of most of the chemicals, though less effectively than MC. Neither inducer affected the activation of 2-AAF by liver microsomes.

Metabolic activation by reconstituted monooxygenase systems containing highly purified P-450s. In a reconstituted monooxygenase system, PB-1, which is a major P-450 form induced by PB, had the highest activity in converting 2,4,2',5'-TCB to protein-bound metabolites, but it was least active in catalyzing 3,4,3',4'-TCB (Table 3). Conversely, MC-1,

Table 3. TCB-binding activities by a reconstituted monooxygenase system containing three forms of purified rat P-450

	TCB-binding activity (pmol/min/nmol P-450)	
	2,4,2',5'-TCB	3,4,3',4'-TCB
PB-1	55.0 ± 2.40	3.72 ± 1.02
MC-1	3.16 ± 1.72	42.5 ± 0.28
MC-2	2.43 ± 1.26	14.7 ± 0.07

Determination of TCB-binding activity by a reconstituted monooxygenase system was carried out as described in Materials and Methods. Each value represents the mean of triplicate determinations ± SD.

Table 4. Activation of *umu* gene expression by procarcinogens and promutagens in a reconstituted monooxygenase system containing three forms of P-450

	Conc (mM)	Activation of *umu* gene expression (units/min/nmol P-450)		
		PB-1	MC-1	MC-2
Trp-P-1	0.01	<10	4200 ± 380	5900 ± 520
Trp-P-2	0.01	90 ± 10	4700 ± 490	5100 ± 650
Glu-P-1	0.01	90 ± 10	500 ± 40	5500 ± 430
IQ	0.01	400 ± 50	1900 ± 200	5400 ± 490
MeIQ	0.01	200 ± 40	2300 ± 150	6500 ± 380
MeIQx	0.01	140 ± 20	1200 ± 110	4100 ± 410
2-AA	0.01	1800 ± 90	1800 ± 200	3000 ± 250
AFB_1	0.01	610 ± 80	1700 ± 120	2000 ± 190
B[*a*]P	0.01	480 ± 40	1400 ± 90	380 ± 70
1,2,3,4-DBA	0.01	190 ± 20	520 ± 50	360 ± 40
2-AAF	0.01	270 ± 30	270 ± 60	470 ± 40
7,12-DMBA	0.01	<10	420 ± 60	<10
MC	0.01	<10	120 ± 20	60 ± 20
AαC	0.08	240 ± 20	2500 ± 150	3300 ± 310
MeAαC	0.08	230 ± 30	2700 ± 250	2300 ± 330

Each value represents the mean of duplicate determinations ± SD.

which is a major form of P-450 induced by MC and has been shown to be a low spin type of P-450, had the highest monooxygenase activity toward 3,4,3′,4′-TCB but had low activity toward 2,4,2′,5′-TCB. MC-2, which is a high spin type of P-450 induced by MC, had considerable activity in converting 3,4,3′,4′-TCB to protein-bound metabolites, though lesser than MC-1. MC-2 was also least active in catalyzing the hydroxylation of 2,4,2′,5′-TCB.

Table 4 compares the abilities of these three forms of rat P-450 to activate fifteen procarcinogens and promutagens to DNA-damaging agents in *umu* test system. In general, the two forms of P-450 induced by MC had more ability to activate these compounds than PB-1. MC-2 was the most efficient catalyst for the following substrates: Trp-P-1, Glu-P-1, IQ, MeIQ, MeIQx, 2-AA, AFB_1, 2-AAF, AαC and MeAαC. On the other hand, the superior substrates

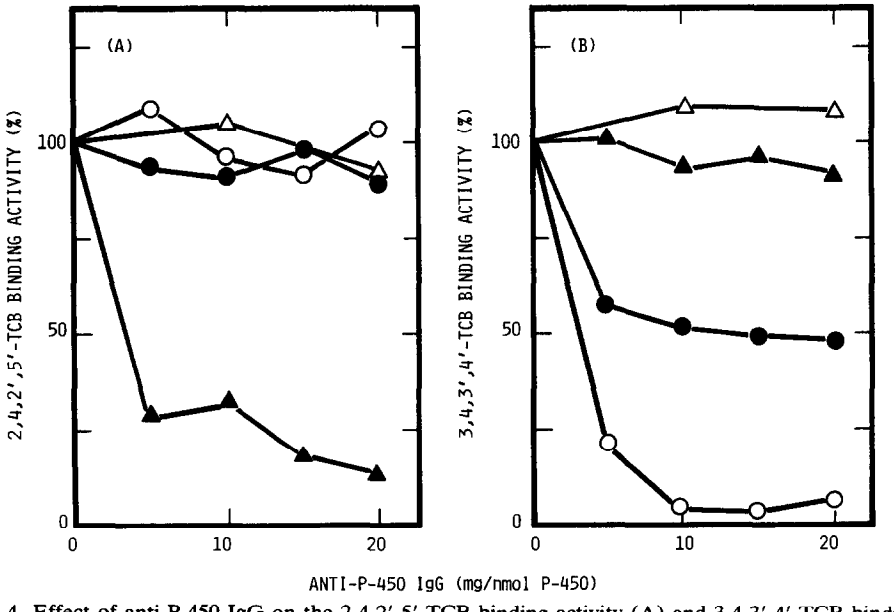

Fig. 4. Effect of anti-P-450 IgG on the 2,4,2′,5′-TCB binding activity (A) and 3,4,3′,4′-TCB binding activity (B) catalyzed by liver microsomes from KC-500-treated rats. The liver microsomes were first incubated with anti-P-450 IgG at 25° for 15 min before adding an NADPH-generating system and the substrates. The reaction was run at 37° for 15 min, and the amount of covalent binding of reactive metabolites of TCBs to proteins was determined as described in Materials and Methods. The activities in the absence of antibodies are shown in Table 1. Effects of control IgG (△), anti-PB-1 IgG (▲), anti-MC-1 IgG (○), and anti-MC-2 IgG (●) are shown. Each point represents the mean of duplicate determinations.

for MC-1 were: 1,2,3,4-DBA, 7,12-DMBA, B[*a*]P and MC. The activities to catalyze Trp-P-2 by MC-1 and MC-2 were almost the same.

Effects of anti-P-450 IgGs on the metabolic activation of procarcinogens and promutagens by liver microsomes. From these results, it was suggested that MC-2 has a major role in metabolic activation of a variety of chemicals examined. To obtain more conclusive evidence, we prepared specific antibodies to three forms of P-450 and used them for the experiments of the effects on metabolic activation of chemicals by liver microsomes from KC-500-treated rats. The specificities of these antibodies were examined by Ouchterlony immunodiffusion analysis and by immunoinhibition of microsomal catalytic activities toward 2,4,2′,5′-TCB and 3,4,3′,4′-TCB. Supporting the results of Kuwahara *et al.* [6], three antibodies did not show any cross-reaction with heterogenous antigens (data not shown). As shown in Fig. 4, the specificities were also confirmed by experiments employing the effects of three IgGs on catalytic activities towards 2,4,2′,5′-TCB and 3,4,3′,4′-TCB by liver microsomes from KC-500-treated rats. As expected, anti-PB-1 IgG inhibited markedly 2,4,2′,5′-TCB-binding activity by liver microsomes, whereas both antibodies to MC-1 and MC-2 did not (Fig. 4A). On the contrary, anti-MC-1 IgG inhibited almost completely the conversion of 3,4,3′,4′-TCB to reactive metabolites that bound covalently to microsomal proteins whereas anti-PB-1 IgG was the least active (Fig. 4B). Anti-MC-2 IgG was also effective in inhibiting 3,4,3′,4′-TCB-binding activity though to a lesser extent than anti-MC-1 IgG.

Figure 5 shows the effects of these antibodies on the metabolic activation of procarcinogens and promutagens catalyzed by liver microsomes from KC-500-treated rats. The substrates examined were nine chemicals which showed high abilities to activate *umu* gene expression after metabolism by liver microsomes (cf Table 2). Anti-MC-2 IgG inhibited very markedly the activation of Trp-P-1, Glu-P-1, IQ, MeIQ, MeIQx, AαC, and 2AA. Anti-MC-1 IgG was also inhibitory for these reactions though less than anti-MC-2 IgG, except for Trp-P-2 and MeAαC. On the other hand, anti-PB-1 IgG did not inhibit the microsome-catalyzed metabolic activation of the chemicals examined. We also examined the effects of these antibodies on the metabolic activation of AFB_1 by liver microsomes of KC-500-treated rats and found that none of the antibodies inhibited the microsome-catalyzed activation of AFB_1 more than 15%.

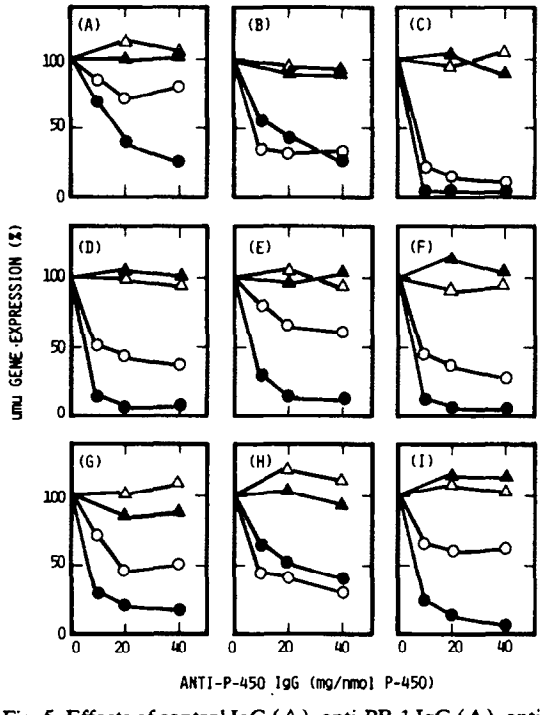

Fig. 5. Effects of control IgG (△), anti-PB-1 IgG (▲), anti-MC-1 IgG (○), and anti-MC-2 IgG (●) on the activation of *umu* gene expression by procarcinogens and promutagens in liver microsomes from KC-500-treated rats. The substrates used were: Trp-P-1 (a), Trp-P-2 (B), Glu-P-1 (C), IQ (D), MeIQ (E), MeIQx (F), AαC (G), MeAαC (H), and 2AA (I). The specific activities in the absence of antibodies are shown in Table 2. Each point represents the mean of duplicate determinations.

DISCUSSION

There are numerous reports which show that a variety of chemicals are carcinogenic and mutagenic; some are alkylating agents themselves, and the others require metabolic activation by several enzymes such as P-450, epoxide hydrolase, glutathione transferase, and *N*-acetyltransferase [2, 3, 21–23]. To detect mutagenic and carcinogenic activities of a number of environmental chemicals, many investigators use the Ames test in the presence or absence of activating enzyme systems [2, 3, 21–23]. Indeed, this method is very useful because of its simplicity, sensitivity, and the close correlation with the results of carinogenicity testing reported so far. It takes, however, about 3–4 days to complete the measurements, requires several bacterial tester strains in the screening of many types of alkylating agents, and is not applicable to some mutagens, including bleomycin, hydrogen peroxide, 5-fluorouracil and paraquat. The newly developed *umu* test system which is one of the SOS function tests to detect DNA-damaging agents has been reported [1]. This system may be more useful than the Ames test with respect to the problems described above, but little information on the kinetics of the reactions or on the possible roles of activating enzymes such as multiple forms of P-450 is available at present.

In this study, we present the following results: (a) the activation of *umu* gene expression by mutagens and carcinogens can be expressed on the basis of unit β-galactosidase activity/min, because the reaction increased linearly between 30 and 90 min of incubation time in both systems containing a directly-acting mutagen, furylfuramide, or an activation-requiring promutagen with liver microsomes, (b) increases in the activation of *umu* gene expression by various procarcinogens and promutagens absolutely depended on the concentrations of P-450 in liver microsomal and reconstituted monooxygenase systems, (c) the substrate specificities of three forms of rat P-450 were determined quantitatively, and (d)

using specific antibodies against three forms of P-450, the importance of individual forms of P-450, particularly a high spin form isolated from MC-treated rats, in the metabolic activations of procarcinogens and promutagens was strongly suggested.

Several lines of evidence suggest that various types of procarcinogens and promutagens are bioactivated to reactive electrophiles by two MC-inducible forms of P-450 more efficiently than by PB-inducible ones in rat liver microsomes [21–26]. Recently, these MC-inducible forms of rat P-450 have been purified, characterized in detail, and cloned in several laboratories [6, 27–31]. Although both forms of P-450 are induced by MC, β-naphthoflavone, and several toxic polyhalogenated compounds simultaneously, they show considerable substrate specificity for metabolism of a wide variety of chemicals [26–28]. In this study we also found diverse effects of two MC-inducible P-450s in activating several procarcinogens and promutagens by measuring induction of *umu* gene expression of *S. typhimurium* TA1535/pSK1002. Good substrates for MC-1, which is also called P-450c and P-450BNF-B [6, 27, 28], were found to be 1,2,3,4-DBA, 7,12-DMBA, B[*a*]P, and MC. On the other hand, Trp-P-1, Trp-P-2, Glu-P-1, IQ, MeIQ, MeIQx, 2-AA, Aα*C* and MeAα*C* were catalyzed most actively by MC-2, which may be the same enzyme as P-450d and P-450ISF-G [6, 27, 28]. Thus, the importance of specific P-450 isozymes to activate procarcinogens and promutagens was ascertained in this *umu* test system. This conclusion was also supported in experiments employing the effects of specific antibodies against PB-1, MC-1, and MC-2 on the reactions catalyzed by liver microsomes from KC-500-treated rats (cf. Fig. 5).

AFB_1 is a hepatocarcinogenic and mutagenic mycotoxin only after metabolic activation by a P-450-linked monooxygenase activity [24, 25, 32]. Several studies on immunoinhibition of catalytic activities have demonstrated that the mutagenic activation of AFB_1 is mediated by both forms of P-450 that are inducible by PB and MC [24, 25]. The present study on the activation of *umu* gene expression by AFB_1 in a reconstituted monooxygenase system confirmed the above findings. It is interesting, however, that liver microsomes of untreated rats had a greater ability to activate AFB_1 than those from PB-, MC-, and KC-500-treated rats. These results suggest that the constitutive form(s) of P-450 as well as the inducible ones may also be involved in the activation of AFB_1. Very slight inhibition of antibodies to PB-1, MC-1, and MC-2 of liver microsome-catalyzed activation of AFB_1 supported the above hypothesis. Our preliminary experiments indicated that constitutive forms of P-450 had profound roles for genotoxic and mutagenic activation of AFB_1 by rat liver microsomes (unpublished results).

N-Hydroxylation of carcinogenic heterocyclic aromatic amines by P-450 is the first step toward the exertion of their carcinogenic and mutagenic activities; these oxidative intermediates require further metabolism by cytosolic or bacterial *N*-hydroxyarylamine *O*-acetyltransferase [19, 20, 26]. All of the eight heterocyclic aromatic amines examined in this study were found to induce *umu* gene expression only after metabolism by P-450. Although all of them were activated strongly by MC-inducible forms of P-450 in the reconstituted system, only MeIQ was catalyzed by liver microsomes of untreated rats more actively than by those from PB-, MC- and KC-500-treated rats (cf. Table 2). However, the findings that anti-MC-1 IgG stronly inhibited the genotoxic activation of MeIQ by liver microsomes supported the view that MC-2 is a major isozyme involved in this reaction.

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