alleles of the *CYP2A6* gene have less risk for tobacco-related lung cancer. Our large-scale epidemiological studies clearly supported this idea (24–26). Based on these results, it was assumed that CYP2A6 determines the cancer risk caused by tobacco-smoking via its ability to activate the nitrosamines. However, it was necessary to further ascertain this idea using experimental animals.

8-Methoxypsoralen is a naturally occurring furanocoumarin derivative found in many plant species such as celery, figs, limes, parsnip and parsley (27). Oral administration of 8-methoxypsoralen, followed by ultraviolet A light, has photosensitizing effects. Thus, this drug has been used clinically for the treatments of psoriasis (28,29). 8-Methoxypsoralen is also known as a potent mechanism-based inhibitor of CYP2A6 and the orthologous forms, mouse CYP2A5 and rat CYP2A3 (30–32). Recently, we demonstrated that lung tumorigenesis caused by NNK was completely suppressed in female A/J mice which had been pretreated with 8-methoxypsoralen (33), probably indicating that 8-methoxypsoralen prevented the occurrence of the lung tumorigenesis by inhibiting the metabolic activation of NNK catalyzed by CYP2As present in the lungs of mice. However, we still needed to provide direct evidence as to whether or not 8-methoxpsoralen inhibited the mutagenic activation of NNK.

Thus, in the present study, we first examined the inhibitory effects of 8-methoxypsoralen on the mutagenic activation of NNK by both liver or lung microsomes from mice and the forms of mouse CYP2A (CYP2A4, CYP2A5 or CYP2A12) expressed in the *Salmonella typhimurium* YG7108. To evaluate the *in vivo* effects of 8-methoxypsoralen on the mutagenicity and tumorigenicity of NNK *in vivo*, we carried out two assays using the *gpt delta* transgenic mice carrying a shuttle vector as a monitor of mutation and using female A/J mice as an experimental animal model for lung tumorigenesis, respectively. Based on results presented in this paper, we propose that suppression of the initiation step of carcinogenesis by pretreatment with CYP2A inhibitors can result in the chemoprevention of lung cancer caused by tobacco smoking.

Materials and methods

Animals and chemicals

Seven-week-old female C57BL/6J mice, 9-week-old female C57BL/6J transgenic mice homozygous for the *gpt delta* gene and 5-week-old female A/J mice (Japan SLC, Shizuoka, Japan) were used. NNK (purity >99%) was purchased from Toronto Research Chemicals (Toronto, Canada). ENU and 8-methoxypsoralen were obtained from Sigma (St Louis, MO). All other chemicals and solvents were of the highest grade commercially available.

Expression of CYP2A in S.typhimurium YG7108 cells

Original *S.typhimurium* YG7108 and three strains of the genetically engineered *S.typhimurium* YG7108 cells, each co-expressing a mouse CYP2A and CPR established previously in our laboratory, were used. The P450 and CPR in the genetically engineered *S.typhimurium* YG7108 cells were expressed according to the method previously described by Fujita *et al.* (34). The content of P450 holo-protein in the *S.typhimurium* cells was determined by Fe^{2+}·CO versus Fe^{2+} difference spectra according to the method of Omura and Sato (35). The expression levels of P450 ranged from 77 nmol/l culture for CYP2A4 to 340 nmol/l culture for CYP2A12. The activity of CPR in sonicated bacterial cells was measured with cytochrome *c* as an electron acceptor by measuring the absorbance change at 550 nm at 20°C according to the method of Philips and Langdon (36). The unit of CPR was defined as the amount of the enzyme that reduced 1 μmol of cytochrome *c*/min. The molar ratio of the expressed CPR to P450 varied from 1.0 for CYP2A12 to 1.9 for CYP2A4.

Mutation assays

The mutation assay with the parental YG7108 cells was performed as described by Maron and Ames (37), with modifications. Briefly, when needed, NNK was metabolically activated outside the *S.typhimurium* YG7108 cells. The effects of the specific inhibitor of P450 forms on the activation of NNK by liver or lung microsomes from mice were examined. The liver and lung microsomes (70 pmol of total P450/reaction) from 7-week-old female C57BL/6J mice were added, which had been suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 1.0 mM ethylenediaminetetraaceticacid and 20% (v/v) glycerol as described previously (38). The parental *S.typhimurium* YG7108 cells were pre-exposed to 100 or 1000 μM of NNK in the presence or absence of various concentrations of specific inhibitors, substrates or antibodies of P450 at 37°C for 20 min before plating. The modifiers employed were α-naphthoflavone (CYP1A), coumarin (CYP2A), 8-methoxypsoralen (CYP2A), chloramphenicol (CYP2B), tolbutamide (CYP2C), quinidine (CYP2D), chlorzoxazone (CYP2E1) and ketoconazole (CYP3A). All inhibitors were dissolved in dimethylsulfoxide to give a final concentration of the organic solvent in the incubation mixtures lower than 1%. Antibodies against rat CYP2A1 raised in rabbits were added to the reaction mixtures (up to 50 μl/mg protein). The mixtures were preincubated at 25°C for 30 min, followed by the addition of NNK as previously described. An NADPH-generating system was added to the reaction mixtures to start incubations.

When the genetically engineered *S.typhimurium* YG7108 was used, NNK was metabolically activated inside the bacterial cells without addition of any other enzymes. In this experiment, the mutagen-producing activity of a form of mouse CYP2As in the activation of NNK was expected to be clarified. The *Salmonella* cells were first pre-exposed to 0.1–250 nM of NNK for 20 min before plating. The NADPH-generating system was not added to the reaction mixtures since the mutagenic activation of promutagens was not affected by the addition of NADPH, probably because NADPH present in the bacterial cells was utilized as an electron donor. The plates were incubated at 37°C for 2 days. Assays were carried out at least twice in duplicate. When the variability of the values of duplicate determinations was within ±20%, the results were adopted. Induced revertants/pmol P450 were calculated based on the increased colonies per pmol of P450 content expressed in the tester strain. The spontaneous revertant number per plate ranged from 20 to 40. Apparent kinetic analysis for the activation of NNK was performed using a computer program (Microcal Origin, Microcal Software, Northampton, MA) designed for non-linear regression analysis. The concentration of 8-methoxypsoralen which decreased the number of revertants to 50% of control (in the absence of 8-methoxypsoralen) was defined as IC_{50}.

Detection of CYP2As in the mouse lung

Total RNAs were prepared from the lung or liver of the 9-week-old female C57BL/6J mouse using RNeasy® Midi kit (QIAGEN, Hilden, Germany). To examine the expression levels of CYP2A mRNA in the mouse tissues, reverse transcriptase–polymerase chain reaction (RT–PCR) was carried out. Total RNA (1 μg) and oligodeoxythymidylic acid primer (0.5 μg) were mixed, incubated at 70°C for 10 min and then cooled on ice. Subsequently, moloney murine leukemia virus reverse transcriptase (20 units) (Toyobo, Tokyo, Japan), RNase inhibitor (20 U) (Takara, Tokyo, Japan), and 0.5 mM, each of four deoxynucleoside triphosphates, were added to the RNA-primer mixtures and then incubated at 42°C for 50 min. PCR was performed in a solution containing cDNA synthesized in the above reaction mixtures (1 μM), 1.5 mM $MgCl_2$, 0.2 mM each of four deoxynucleoside triphosphates, each primer (1 μM), AmpliTaq Gold polymerase (2.5 U) (PerkinElmer Life Sciences) and 10× AmpliTaq reaction buffer (5 μl) (PerkinElmer Life Sciences). PCR was carried out under the following conditions: 30 cycles of reactions composed of cycle denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The PCR products (96 bp for CYP2A4 and CYP2A5 and 143 bp for CYP2A12) were subjected to a 2% agarose gel and then visualized by ethidium bromide staining. The sequences of primers used are as follows: CYP2A4, 5′-GCCAGCTCTATGAGATGTTT-3′ and 5′-TTATAAAGTCCTCCAGGCCT-3′; CYP2A5, 5′-GCCAGGTCTATGAGATGTTT-3′ and 5′-TTATAAAGTCCTCCAGGCCC-3′; CYP2A12, 5′-TTAGCTCCATTGTCTTCGGC-3′ and 5′-GGTACTTCATAACTGAATGG-3′; β-actin, 5′-ATTGCTGACAGGATGCAGA-3′ and 5′-GCTCAGGAGGAGCAATGATCTT-3′.

gpt mutation assay

The *gpt delta* C57BL/6J transgenic mice were maintained in the Animal Facility of the Faculty of Pharmaceutical Sciences, Hokkaido University, according to the institutional animal care guidelines. The animals were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity (55 ± 5%), lighting (12 h light/dark cycle) and temperature (23 ± 1°C). The animals were pretreated with 8-methoxypsoralen at a dose of 12.5 or 50 mg/kg body wt in 0.2 ml of corn oil or an equal volume of corn oil as a vehicle control via stomach tube daily for 7 days. One hour after the third treatment, the animals were treated with a single i.p. injection of NNK or ENU at a dose of 100 mg/kg body wt or an equal

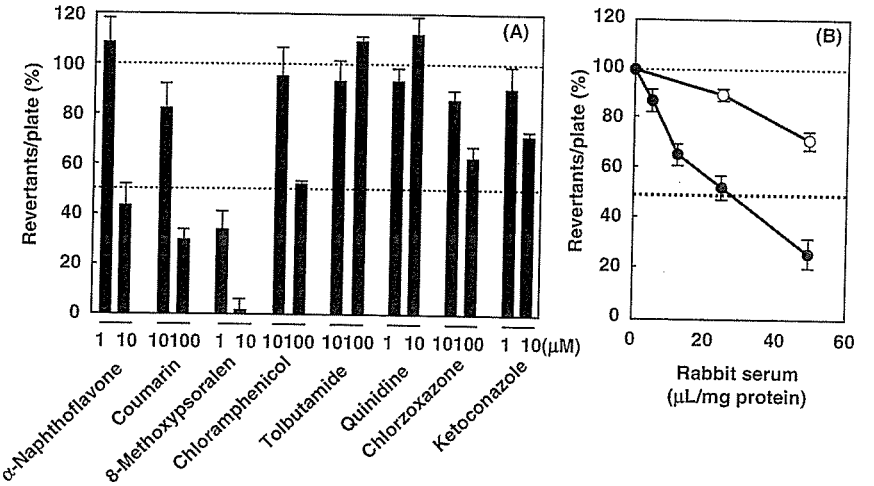

Fig. 1. Effects of specific inhibitors of forms of P450 (A) and anti-CYP2A1 antibodies (B) on the mutagenic activation of NNK catalyzed by mouse liver microsomes. The number of NNK-induced revertants without the inhibitors (A) or sera (B) was 103 revertants/plate. (A) The inhibitors employed were α-naphthoflavone (for CYP1A), coumarin (CYP2A), 8-methoxypsoralen (CYP2A), chloramphenicol (CYP2B), tolbutamide (CYP2C), quinidine (CYP2D), chlorzoxazone (CYP2E1) and ketoconazole (CYP3A). (B) Open and closed circles represent the results with rabbit antiserum to rat CYP2A1 and preimmune serum, respectively. Each value and bar indicates the mean and SD (range) of duplicate determinations, respectively.

volume of saline as a vehicle control. Two weeks after the final treatment, mice were killed under ether anesthesia. They were quickly frozen in liquid nitrogen and stored at −80% until analysis. The *gpt* mutagenesis assay was performed as previously described (39).

Tumorigenicity test

Female A/J mice were maintained in the Animal Facility of the Faculty of Medicine, Kagawa University, according to the institutional animal care guidelines. The animals were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast), under controlled conditions of humidity at 60 ± 10%, lighting with 12 h light/dark cycle and temperature at 24 ± 2°C.

The animals were pretreated with 8-methoxypsoralen at a dose of 0.125, 1.25 or 12.5 mg/kg body wt in 0.2 ml corn oil or an equal volume of corn oil as a vehicle control via stomach tube, daily for 3 days. One hour after the last pretreatment, animals were given a single dose of NNK at a dose of 100 mg/kg body wt in 0.1 ml saline via p.o. or an equal volume of saline as a vehicle control. The experiment was terminated at 16 weeks after the first 8-methoxypsoralen pretreatment. In separate experiments, three daily doses of 8-methoxypsoralen (12.5 mg/kg) were given to mice 1, 3 and 7 days after a single dose of NNK injection. At autopsy, their lungs were excised and weighed, infused with 10% neutral buffered formalin and carefully inspected grossly. All of the macroscopically detected lung nodules were counted, and each lung lobe was examined histopathologically. Lung lesions, hyperplasias and adenomas were diagnosed according to the criteria of 'Tumors of the Mouse' (40), and the number of hyperplasias and adenomas was counted under a microscope.

Immunohistochemistry

Lungs were immunostained for CYP2A by the ABC method, all staining processes from deparaffinization to counterstaining with hematoxylin being performed automatically using the Ventana Discovery™ staining system (Ventana Medical Systems, AZ, USA). Anti rabbit CYP2A1 polyclonal antibodies were used at 1:50 dilution.

Statistical analysis

The incidence of lung proliferative lesions were analyzed by the Fisher's exact probability test and data for multiplicity by Student's *t*-test.

Results

Effects of CYP2A inhibitors and anti-CYP2A antibodies on the mutagenic activation of NNK catalyzed by liver and lung microsomes from mice

To estimate a possible P450 form(s) responsible for the mutagenic activation of NNK in mice, effects of the representative

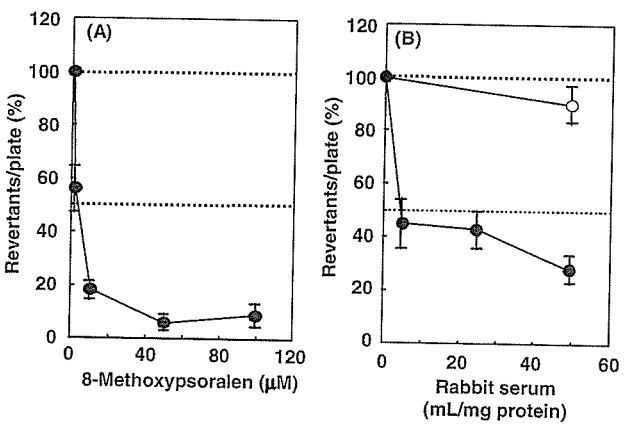

Fig. 2. Inhibition by 8-methoxypsoralen (A) and antiserum to rat CYP2A1 (B) of the mutagenic activation of NNK catalyzed by mouse lung microsomes. The number of NNK-induced revertants without 8-methoxypsoralen (A) and sera (B) was 85 revertants/plate. See legend of Figure 1 for details. In the presence of methanol as a solvent control (A), the number of NNK-induced revertants was 80 revertants/plate.

inhibitors of P450s and anti-P450 antibodies on the genotoxic activation of NNK catalyzed by liver and lung microsomes from mice were examined. Among the inhibitors tested, coumarin and 8-methoxypsoralen, the inhibitor of CYP2A, efficiently inhibited the mutagenic activation of NNK catalyzed by liver microsomes (Figure 1A). Anti-CYP2A1 antibodies efficiently inhibited the activation of NNK by mouse liver microsomes (Figure 1B).

As was seen with liver microsomes, 8-methoxypsoralen and anti-CYP2A1 antibodies inhibited the mutagenic activation of NNK catalyzed by lung microsomes in a dose-dependent manner (Figure 2).

Metabolic activation of NNK by mouse CYP2As expressed in S.typhimurium YG7108

To determine which one of the mouse CYP2A forms is responsible for the mutagenic activation of NNK, we

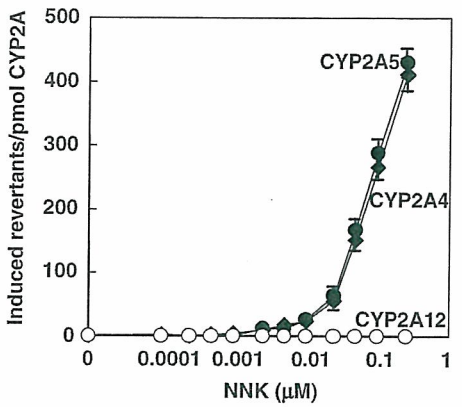

Fig. 3. Mutagenic activation of NNK by CYP2A4, CYP2A5 and CYP2A12 expressed in *S.typhimurium* YG7108 cells. NNK (0.1–250 nM) was exposed to *S.typhimurium* YG7108 cells expressing a mouse CYP2A. Closed diamond, closed circle and open circle represent the results with *S.typhimurium* YG7108 cells expressing CYP2A4, CYP2A5 and CYP2A12, respectively. Each value and bar indicates the mean and SD (range) of duplicate determinations, respectively.

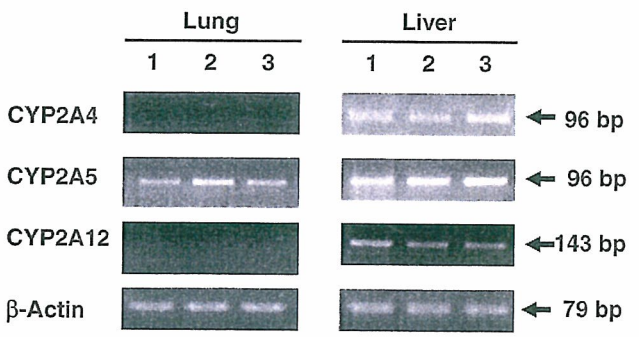

Fig. 4. Expression levels of mRNAs for CYP2A4, CYP2A5 and CYP2A12 in mouse lungs. Total RNA was prepared from lungs and livers of female mice. The expression of mRNAs for CYP2A4, CYP2A5 and CYP2A12 in the mouse lung or liver was determined by RT–PCR as described in Materials and methods. The sequence of specific primers for the mouse CYP2As is also described under Materials and methods. One tenth of each PCR solution was applied and separated in a 2% agarose ethidium bromide-stained gel. Each lane represents three individual samples.

performed the mutation assays using the genetically engineered *S.typhimurium* YG7108 each expressing CYP2A4, CYP2A5 or CYP2A12 together with CPR. As shown in Figure 3, CYP2A4 and CYP2A5, but not CYP2A12, was involved in the mutagenic activation of NNK. Apparent kinetic parameters for the mutagenic activation of NNK by CYP2A4 and CYP2A5 were calculated from the standard Michaelis–Menten velocity equation. Apparent K_m and V_{max} values for the activation by CYP2A4 and CYP2A5 were estimated to be 0.17 ± 0.03 μM and 770 induced revertants/pmol CYP2A4/μM and 0.18 ± 0.04 μM and 750 induced revertants/pmol CYP2A5/μM, respectively. Thus, both CYP2A forms showed similar apparent V_{max}/K_m values (4500 for CYP2A4 and 4200 for CYP2A5).

However, examination for the expression of CYP2As in mouse lung by RT–PCR indicated that mRNA for CYP2A5, but not for CYP2A4, was expressed, as shown in Figure 4. Taken together, these results suggested that CYP2A5 was a principal enzyme responsible for the mutagenic activation of NNK in the mouse lung.

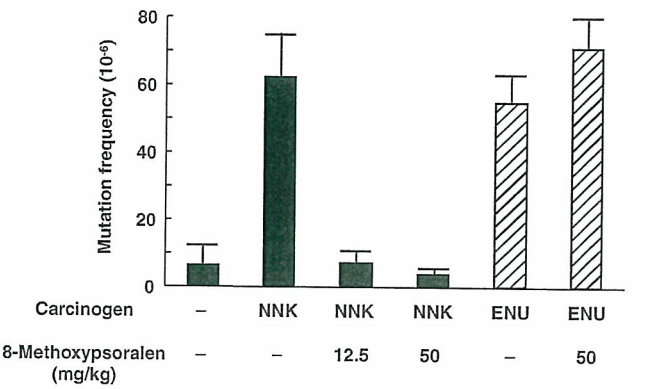

Fig. 5. 8-Methoxypsoralen inhibits the NNK-induced, but not ENU-induced, mutation of the *gpt delta* gene of the lung of *gpt delta* transgenic mice. Mice were pretreated with 8-methxypsoralen at a dose of 12.5 or 50 mg/kg body wt per oral or an equal amount of corn oil as a vehicle control with a stomach tube daily for 7 days. One h after treatment of mice with 8-methxypsoralen on day 3, each animal was treated with NNK or ENU at a dose of 100 mg/kg body wt, i.p. or equal volume of saline once daily for 4 days. The experiment was terminated on day 14 after the final NNK treatment. Each value and bar indicates the mean and SD (range) of duplicate determinations, respectively.

Effects of 8-methoxypsoralen on in vivo mutation caused by NNK in the gpt delta transgenic mice

To elucidate the role of mouse CYP2A in NNK-induced *in vivo* mutagenesis, we assessed the effects of 8-methoxypsoralen against NNK-induced mutation by using the transgenic mice carrying the *gpt delta* gene as a monitor of mutation. Oral feeding of 8-methoxypsoralen did not result in any apparent toxic effects even at a high concentration of 8-methoxypsoralen (100 mg/kg). No significant change was seen in food and water consumption and body wt (data not shown). As shown in Figure 5, the frequency of spontaneous mutation in the *gpt delta* gene in the lung was 6.4×10^{-6}. A single i.p. injection of NNK resulted in a 10-fold higher frequency of mutation (62.2×10^{-6}). The NNK-induced mutations in the lung were reduced by 89 and 94% when mice were pretreated with 8-methoxypsoralen at a dose of 12.5 or 50 mg/kg, respectively, prior to NNK. On the other hand, pretreatment of mice with 8-methoxypsoralen did not reduce ENU (a direct mutagen)-induced mutation whereas treatment of mice with ENU resulted in a 9-fold higher frequency of mutation (55.0×10^{-6}) in the *gpt* gene of the lung.

Effects of 8-methoxypsoralen on the NNK-induced lung tumors in A/J mice

As described previously, our previous study had demonstrated that pretreatment of female A/J mice with 8-methoxypsoralen strongly inhibited NNK-induced lung tumorigenesis (33). Here, we further investigated the inhibitory effects of 8-methoxypsoralen on the NNK-induced lung tumorigenesis. Lung whitish nodules were readily detected in the group of mice treated with NNK alone macroscopically but were rare in the group of mice treated with 8-methoxypsoralen alone and the group of mice pretreated with 12.5 mg/kg of 8-methoxypsoralen + NNK (Table I). Lung carcinoma could not be seen in any of the animals. Numbers and/or incidence of macroscopically observed lung nodules induced by NNK were reduced by 8-methoxyapsoralen in a dose-dependent manner ($P < 0.01$). The numbers of tumors/mouse were reduced from

21.04 (NNK alone) to 17.05, 7.89 and 1.25 by 0.125, 1.25 and 12.5 mg/kg body wt of 8-methoxyapsoralen, respectively, with the inhibitions by the latter two doses being statistically significant ($P < 0.01$).

The multiplicities and incidence of lung hyperplasias and adenomas are also summarized in Table I. Numbers and/or incidence of microscopically observed adenoma and hyperplasia induced by NNK were also reduced by 8-methoxypsoralen in a dose-dependent manner. Numbers of adenoma and hyperplasia were reduced from 15.61 (NNK alone) to 11.90, 5.44 and 1.15 by 0.125, 1.25 and 12.5 mg/kg body wt of 8-methoxypsoralen, respectively (Table I). Similarly, adenoma numbers were reduced from 13.44 to 10.25, 4.44 and 0.85 by 0.125, 1.25 and 12.5 mg/kg body wt of 8-methoxypsoralen, respectively, with the inhibitions by the latter two doses being statistically significant ($P < 0.01$).

To examine the mechanism(s) for the suppression of the NNK-induced lung tumorigenesis by 8-methoxypsoralen, we determined whether or not treatment of mice with 8-methoxypsoralen after the administration of NNK also inhibited the NNK-induced lung tumorigenesis. As shown in Table II, pretreatment of mice with 8-methoxypsoralen inhibited the incidence and multiplicities of macroscopically and microscopically examined lung lesions. However, treatment with 8-methoxypsoralen on days 1, 3 and 7 after NNK administration did not affect the incidence and multiplicities of examined lung lesions, suggesting that 8-methoxypsoralen abolished NNK-induced lung tumorigenesis via the inhibition of initiation event in carcinogenesis but not subsequent events including promotion and progression in carcinogenesis.

Expression of CYP2A in mouse lung adenomas induced by NNK

It was of interest to know if CYP2As were expressed in the region of NNK-induced lung lesions. Thus, we performed immunohistochemical examination for the expression of CYP2A using rabbit polyclonal anti-rat CYP2A1 antiserum in paraffin-embedded tumors containing NNK-induced lung adenomas. As shown in Figure 6, the expression of a protein(s) cross-reactive to antibodies to CYP2A1 was clearly seen in the region of NNK-induced lung tumor. Together with the data shown in Figure 3, it seemed possible to assume that CYP2A5 protein was expressed in the region of NNK-induced lung adenomas.

Discussion

The lung is one of the major target organs for NNK-induced tumor formation in laboratory animals including mice, rats and hamsters, regardless of the route of administration (1). A number of reports have appeared to date indicating that P450 is responsible for the metabolic activation of NNK (1,33,41–44). P450 forms belonging to the CYP2A subfamily have been studied in rats (CYP2A1, CYP2A2 and CYP2A3), mice (CYP2A4 and CYP2A5) and hamsters (CYP2A8, CYP2A9 and CYP2A16). CYP2As from these laboratory animals differ markedly in catalytic specificity despite similarity in their amino acid sequences (~70–90%) (45–50). In the present study, among mouse CYP2As, recombinant CYP2A4 and CYP2A5 activated NNK efficiently at nM concentrations of NNK in the mutation assays using the *S.typhimurium* YG7108, each expressing a form of the mouse CYP2A along with CPR (Figure 3). To our knowledge, the mutagenicity of *N*-nitrosamines including NNK at the nM level could not be detected in other studies reported so far. Furthermore, in our preliminary study, rat CYP2A3 and hamster CYP2A16 also activated NNK at nM levels of NNK using our genetically engineered *S.tyhimurium* YG7108 expressing a CYP2A. Collectively, these data may suggest that CYP2A subfamily member(s) play an important role in the mutagenic activation of NNK.

The mutagenic activation of NNK by mouse liver and lung microsomes was also inhibited by anti-CYP2A1 antibodies (Figures 1B and 2B). Since 8-methoxypsoralen is reported to inhibit CYP2A5 and human CYP2A6-mediated coumarin 7-hydroxylation, it has been recognized as a representative inhibitor of CYP2A5 and CYP2A6 (31,51,52). It has also been shown that 8-methoxypsoralen is a substrate for CYP2A6 and that the enzyme inhibition is due to competitive interaction (53,54). The metabolism-dependent inactivation of CYP2A6 by 8-methoxypsoralen has been shown to occur at low concentrations and at high rates (54). Using the *S.typhimurium* YG7108 expressing high levels of mouse or human CYP2A, 8-methoxypsoralen inhibited the metabolic activation of NNK (IC_{50} values were 0.039–0.66 μM) (unpublished data). The results of the present study provided strong evidence that the suppression by 8-methoxypsoralen, a typical CYP2A inhibitor, of NNK-induced lung tumorigenesis in mice can be explained by the inhibitory effects of 8-methoxypsoralen on the mutagenic activation of NNK catalyzed by CYP2A5 in the target organ in the initiation events of carcinogenesis.

von Pressentin *et al.* (55) have also reported that the administration of NNK in drinking water resulted in an increased mutation frequency in the organ including the lung of the *lacZ* mice (Muta™Mouse). In the present study, NNK-induced mutations in the lung of *gpt delta* transgenic mouse were almost completely abolished when mice were pretreated with 8-methoxypsoralen at a dose of 12.5 mg/kg. On the other hand, pretreatment of mice with 8-methoxypsoralen did not reduce the ENU (a direct mutagen)-induced mutation, suggesting that 8-methoxypsoralen might reduce the NNK-induced gene mutation via the inhibition of metabolic activation of NNK. Our preliminary DNA microarray analyses using the lungs from 8-methoxypsoralen and/or NNK-treated mice have shown that treatment with 8-methoxypsoralen and/or NNK did not alter the expression levels of P450s, phase II enzymes and DNA repair enzymes at 1 day after the treatment (unpublished data), when the metabolic activation of NNK had been reported to be almost completely finished (56). The expression of mRNA for CYP2A5, but not for CYP2A4, in the lung from untreated mice (Figure 4) and CYP2A proteins cross-reactive to anti-CYP2A1 antibodies seen in mouse lung adenoma induced by NNK (Figure 6) probably suggested that 8-methoxypsoralen inhibited further occurrence of mutation in the adenomas induced by NNK.

Treatment of mice with 8-methxypsoralen after NNK administration did not affect the incidence and multiplicities of lung lesions (Table II), probably suggesting that antimutagenic activity of 8-methoxypsoralen against NNK-induced mutation in an initiation event in carcinogenesis is a key step in the chemoprevention of this compound against NNK-induced lung tumorigenesis in mice. We also reported that CYP2A6 was capable of activating betel quid-specific *N*-nitrosamines (57) and that the *CYP2A6* gene deletion reduced oral cancer risk in betel quid chewers in Sri Lanka (58). Taken together with the data of this study, it is reasonable

Table I. Effects of treatment of female A/J mice with 8-methoxypsoralen prior to NNK on NNK-induced lung tumorigenesis

Group	NNK	8-Methoxypsoralen (mg/kg)	No.[a]	Macroscopical lung lesion		Hyperplasia		Adenoma		Hyperplasia and adenoma	
				Incidence (%)[b]	Whitish nodule/mouse[c]	Incidence (%)[b]	Tumors/mouse[c]	Incidence (%)[b]	Tumors/mouse[c]	Incidence (%)[b]	Tumors/mouse[c]
1	−	12.5	20	5/20 (25)	0.25 ± 0.44	1/20 (5)	0.05 ± 0.22	1/20 (5)	0.05 ± 0.93	2/20 (10)	0.10 ± 0.31
2	+	—	23	23/23 (100)	21.04 ± 12.56	18/23 (78)	2.17 ± 1.95	23/23 (100)	13.44 ± 7.94	23/23 (100)	15.61 ± 8.47
3	+	0.125	20	20/20 (100)	17.05 ± 15.24	14/20 (70)	1.65 ± 1.60	20/20 (100)	10.25 ± 7.87	20/20 (100)	11.90 ± 8.92
4	+	1.25	18	18/18 (100)	7.89 ± 2.63[d]	11/18 (61)	1.00 ± 0.97	18/18 (100)	4.44 ± 1.82[d]	18/18 (90)	5.44 ± 2.12[d]
5	+	12.5	20	12/20 (60)[e]	1.25 ± 1.29[d]	6/20 (30)[e]	0.30 ± 0.47	12/20 (60)[e]	0.85 ± 0.93[d]	14/20 (70)[e]	1.15 ± 1.04[d]

NNK (100 mg/kg) was injected i.p. to mice as described in Materials and methods.
[a]Number of mice examined.
[b]Number of mice with lesion (%).
[c]Mean ± SD.
[d]Significantly different from Group 2 by Student's *t*-test ($P < 0.01$).
[e]Significantly different from Group 2 by Fisher's exact probability test ($P < 0.01$).

Table II. Effects of treatment of female A/J mice with 8-methoxypsoralen before or after the administration of NNK on NNK-induced lung tumorigenesis

Group	NNK	8-Methoxypsoralen (mg/kg)	No.[a]	Macroscopical lung lesion		Hyperplasia		Adenoma		Hyperplasia and adenoma	
				Incidence (%)[b]	Whitish nodule/mouse[c]	Incidence (%)[b]	Tumors/ mouse[c]	Incidence (%)[b]	Tumors/mouse[c]	Incidence (%)[b]	Tumors/mouse[c]
1	−	Alone	19	3/19 (16)	0.16 ± 0.38	0/19 (0)	0	2/19 (11)	0.11 ± 0.32	2/19 (11)	0.11 ± 0.32
2	+	—	20	19/20 (95)	3.70 ± 3.06	9/20 (45)	0.75 ± 1.07	18/20 (90)	2.75 ± 2.10	19/20 (95)	3.50 ± 2.42
3	+	Pretreatment	20	9/20 (45)[d]	0.95 ± 1.54[e]	4/20 (20)	0.50 ± 1.15	8/20 (40)[d]	0.60 ± 0.88[e]	8/20 (40)[d]	1.10 ± 1.80[e]
4	+	1 day after NNK	19	17/19 (90)	4.26 ± 2.98	11/19 (58)	1.00 ± 1.15	17/19 (90)	3.00 ± 1.73	17/19 (90)	4.00 ± 2.45
5	+	3 days after NNK	20	20/20 (100)	3.70 ± 1.84	10/20 (50)	0.50 ± 0.51	19/20 (95)	3.05 ± 1.96	20/20 (100)	3.55 ± 2.01
6	+	7 days after NNK	20	19/20 (95)	3.10 ± 1.71	8/20 (40)	0.40 ± 0.50	19/20 (95)	2.65 ± 1.73	20/20 (100)	3.05 ± 1.79

NNK (100 mg/kg) was injected i.p. into mice.
[a]Number of mice examined.
[b]Number of mice with lesion (%).
[c]Mean ± SD.
[d]Significantly different from Group 2 by Fisher's exact probability test ($P < 0.01$).
[e]Significantly different from Group 2 by Student's *t*-test ($P < 0.005$).

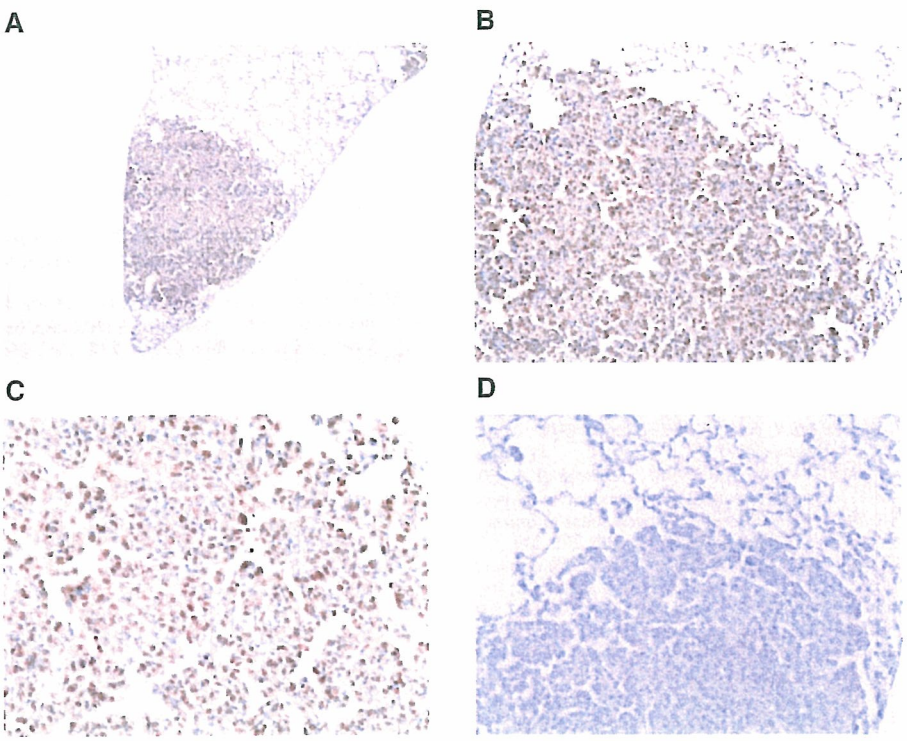

Fig. 6. Immunohistochemical examination for the expression of CYP2A in adenoma induced by NNK in the mouse lung. The boundary of the lesion was usually well demarcated. The paraffin-embedded lung sections were immunostained with rabbit polyclonal anti-rat CYP2A1 antibodies (**A–C**) or preimmune (**D**). A, B and D, and C display lung adenomas at 40×, 100× and 200× magnifications, respectively.

to assume that the function of CYP2A6 as an initial event in carcinogenesis plays an important role in lung cancer caused by smoking. In accordance with this idea, we demonstrated in a previous report that the function of CYP2A6 evaluated from *CYP2A6* genetic polymorphisms is a key determinant of tobacco-related lung cancer risk in male Japanese smokers (26).

In conclusion, the results of this study indicate for the first time that 8-methoxypsoralen prevents NNK-induced lung mutagenesis and tumorigenesis in mice by inhibition of CYP2A5 in the lung.

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Novel Gender-Related Regulation of *CYP2C12* Gene Expression in Rats

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The expression of CYP2C12 by GH occurs in female but not in male rat livers. Direct injection of the *CYP2C12* promoter-luciferase gene into male rat livers showed that the *CYP2C12* promoter was active in both male and female rats. Thus, to further examine one or more factors that regulate the gender-related expression of CYP2C12, male rats were treated with trichostatin A, a specific inhibitor of histone deacetylase capable of condensing the chromatin structure. Interestingly, the expression of CYP2C12 by GH was seen even in the livers of male rats, indicating that histone deacetylase contributes to the suppression of CYP2C12 expression in male rats. Deoxyribonuclease I hypersensitive assay using nuclei from the livers of male or female rats revealed that the chromatin structure of the *CYP2C12* gene was gender specific: a hypersensitive site at a position −4.2 kb containing GH-responsive element that bound to signal transducer and activator of transcription 5 (STAT5), termed as HS (hypersensitive site) 1, was specific for female rat livers, whereas a hypersensitive site at a position −3 kb, designated as HSm (male-specific hypersensitive site), was characteristic of male rat livers. A −3425/−3275 region within HSm functioned as a negative regulatory region, when the region was inserted in front of simian virus 40 promoter. Gel shift assay demonstrated that both CCAAT/enhancer-binding protein α and β bound to the −3425/−3275 region. Based on these results, we conclude that the gender-related expression of the *CYP2C12* gene results from the inaccessibility of to STAT5 to the GH-responsive element by chromatin condensation seen in male rat livers, and from the presence of the male-specific HSm that acts as a silencer. (*Molecular Endocrinology* 19: 1181–1190, 2005)

CYP2C12 IS KNOWN to be one of the steroid hydroxylases and is constitutively expressed in female rats but not in male rats (1–4). The gender-related expression of the *CYP2C12* gene is regulated by GH (5–12). The pattern of GH secretion in male rats is characterized by the peaks of large amplitude every 3–4 h with undetectable levels in interpulse periods, whereas the pattern of GH secretion in female rats shows more frequent oscillation of small amplitude (13, 14). Thus, the transcription of the *CYP2C12* gene is considered to be stimulated by the relatively constant level of GH secreted in female rats (9, 10, 12).

The effects of GH are mediated by a GH receptor, which belongs to a cytokine/hematopoietin receptor superfamily (15). Based on a proposed pathway for the induction of gene expression by GH, the binding of GH to the GH receptor promotes the interaction of the GH receptor with Janus kinase 2 and the tyrosyl phosphorylation of the Janus kinase 2 (16). Subsequently, the activated Janus kinase 2 phosphorylates the tyrosine residues of the signal transducer and activator of transcription (STAT) protein. After the formation of the homodimer of the STAT protein or the heterodimer of the STAT protein with other factor(s) in the cytoplasm, the complex translocates to the nucleus, and then binds to target sequences (17). Among the STAT family, STAT1, STAT3, and STAT5 have been identified as GH-stimulated proteins (15, 18). Particularly, STAT5 (19) has been reported to regulate the GH-related expression of several genes including c-*fos* (20), serine protease inhibitor 2.1 (21) and acid-labile subunit (22). Pulsatile plasma GH secretion seen in male rats but not in female rats has been shown to activate STAT5 in the liver (23, 24). Thus, STAT5 has been regarded as the male-specific regulator for the expression of genes including sex-limited protein (25) and *CYP3A10* (26).

Until now, the one or more mechanisms responsible for the GH-dependent activation of the *CYP2C12* gene in females has been studied *in vitro*. Hepatocyte nuclear factor (HNF)-6 has been reported to participate in the GH-dependent transcription of the *CYP2C12* gene (27). Additionally, it has been reported that the female-enriched GH-dependent

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Abbreviations: Brg1, Brahma-related gene; CBP, CREB-binding protein; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP response element binding protein; DNase, deoxyribonuclease; GHRE, GH-responsive element; HDAC, histone deacetylase; HNF, hepatocyte nuclear factor; HS, hypersensitive site; Oct-1, Octamer transcription factor-1; SNF, sucrose nonfermenting; STAT, signal transducer and activator of transcription; SV40, simian virus 40; SWI, switching defective; TSA, trichostatine A.

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complex, termed as GH nuclear factor, bound to five distinct regions within the *CYP2C12* promoter region between nucleotides −1560 and +60 (28). Recently, we developed the direct DNA injection method to clarify the mechanism involved in the GH-induced expression of the *CYP2C12* gene in female rats. By using the *in vivo* method, we provided lines of evidence that the GH-dependent and liver-specific expression of the *CYP2C12* gene in female rats was caused by a cooperative regulation with STAT5, HNF-4, HNF-6, and factors that bound to elements, 2C12-I and 2C12-II, in the upstream region of the *CYP2C12* gene (29).

STAT5b has been reported to be a key factor to regulate the gender-related expression of the *CYP2C12* gene (30). Cotransfection of HepG2 cells with STAT5b and HNF-6 expression plasmids showed that STAT5b inhibited the transcriptional activation of the *CYP2C12* gene by HNF-6 (30). However, this result does not agree with our *in vivo* data that STAT5b is an essential factor for the transcriptional activation of the *CYP2C12* gene in female rats (29). Thus, it was necessary to clarify whether or not STAT5b is a critical factor determining the gender-related expression of the *CYP2C12* gene *in vivo*. In addition, the true mechanism(s) for the gender-related expression of the *CYP2C12* gene remains to be clarified.

Our hypothesis to explain the mechanism underlying the gender-related expression of the *CYP2C12* gene was as follows. If the female-specific expression of the *CYP2C12* gene is regulated at chromatin level by STAT5, HNF-4, HNF-6, and factors that bound to elements, 2C12-I and 2C12-II, the transcription factors necessary for the transcriptional activation of the *CYP2C12* gene may not bind to their target elements in male rat livers. To prove our hypothesis, we performed deoxyribonuclease (DNase) I hypersensitive assay using nuclei prepared from the livers of male and female rats. In the present paper, we propose that the female-specific expression of the *CYP2C12* gene results from the inaccessibility of STAT5 and basal transcription factors to their target elements by chromatin condensation seen in male rat livers but not in female rat livers.

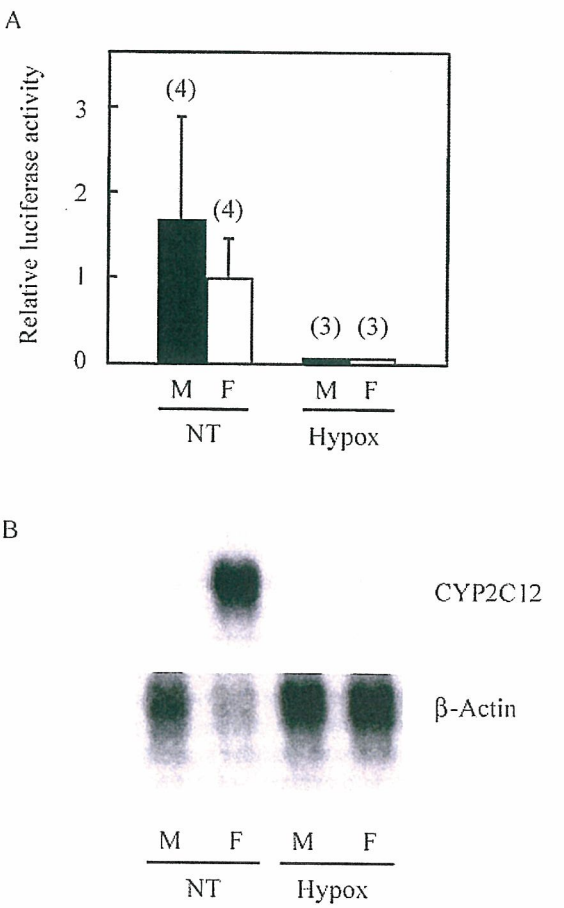

Fig. 1. Gender-Related Expression of the *CYP2C12* Gene in Rats

A, Luciferase activity in the liver, which received Luc10kb. A reporter plasmid Luc10kb was injected into the livers of nontreated and hyphophysectomized female rats. Luc10kb was also injected into the livers of nontreated and hyphophysectomized male rats. All values represent the mean ± SD from independent experiments shown in *parentheses*. The data are expressed as the ratio of the luciferase activity in each rat to the basal activity obtained in nontreated female rats. B, Northern blot analysis of mRNAs for CYP2C12 in the liver treated with DNA. A portion (20 μg) of total RNA was allocated to Northern blot analysis as described in *Materials and Methods*. M, Male; F, female; Hypox, hyphophysectomized; NT, nontreatment.

RESULTS

Direct Injection of the CYP2C12 Gene into Male and Female Rat Livers

To examine whether or not Luc10kb reporter gene possessing the 5′-flanking region up to −10 kb of the *CYP2C12* gene fused to the luciferase gene was activated by GH, we injected the DNA of Luc10kb into the liver of male or female rats after treatment with GH in a manner mimicking the female-specific pattern (Fig. 1A). The result showed that Luc10kb was activated by GH in the livers from both male and female rats. Hypophysectomy of male and female rats resulted in the abolishment of the luciferase activity. Thus, no female-specific activation of Luc10kb was seen in rats with respect to *trans*-acting factors necessary for the GH-dependent activation of Luc10kb reporter gene. To examine the expression level of endogenous CYP2C12 mRNA, Northern blot analysis using total RNAs prepared from liver lobes, which had received Luc10kb, was performed (Fig. 1B). CYP2C12 mRNA was expressed in females but not in males. The expression of CYP2C12 mRNA was lost by hypophysectomy of female rats. These results suggest that the female-

specific expression of the *CYP2C12* gene cannot be fully explained solely by *trans*-acting factors.

Effects of Trichostatine A (TSA) on the Expression of CYP2C12 mRNA in the Liver of Male Rats

Based on the results mentioned above, we hypothesized as follows: if the gender-related expression of the *CYP2C12* gene is regulated by chromatin structure rather than *trans*-acting factors, the accessibility of *trans*-acting factors to their target elements would be different between male and female rat livers. It has been reported that histone deacetylase (HDAC) is one of key factors that condense the chromatin structure (31). Thus, we performed Northern blot analysis using total RNAs from the livers of male rats treated with GH and TSA, which is known to be a specific inhibitor of HDAC (Fig. 2). Interestingly, CYP2C12 mRNA was expressed at efficient rate even in the livers of male rats after treatment with TSA. However, treatment of male rats with TSA alone did not increase the expression level of CYP2C12 mRNA. Although CYP2C12 mRNA was expressed by continuous infusion of GH to male rat livers, the expression level of CYP2C12 mRNA in male rats treated with GH alone was much lower than in female rats. Thus, the results suggest that HDAC contributes to the chromatin condensation of the *CYP2C12* gene, probably resulting in the depression of CYP2C12 expression, in male rat livers.

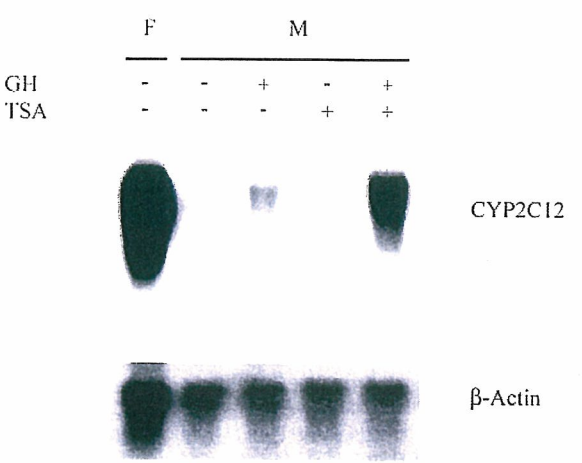

Fig. 2. Effects of TSA on the Expression of CYP2C12 mRNA in the Liver of Male Rats

Male rats were treated with TSA (0.1 mg/kg·d) by a continuous infusion with an osmotic minipump for 24 h. After treatment with TSA, human GH (0.94 IU/kg·d) was given to the same animals by a continuous infusion, which mimics female-type GH secretion, for 7 d. A portion (20 μg) of total RNA was allocated to Northern blot analysis as described in *Materials and Methods*. F, Female; M, male.

DNase I Hypersensitive Sites in the 5′-Flanking Region of the *CYP2C12* Gene

To determine the accessibility to target elements of *trans*-acting factors necessary for the GH-induced expression of the *CYP2C12* gene, DNase I hypersensitive assay using nuclei from the livers of male or female rats was carried out (Fig. 3A). The 5′-flanking region from the nucleotides −927 to −359 of the *CYP2C12* gene was used as a probe (Fig. 3B). The expected size of a fragment protected from DNase I was 6.4 kb, when genomic DNA from DNase I-treated nuclei was digested with *Hin*dIII (Fig. 3, A and B). We found three hypersensitive sites at positions −4.2, −1.2, and −0.7 kb, termed as HS1, HS2, and HS3, respectively, with nuclei from the livers of female rats but not from the livers of male rats. Alternatively, a male-specific hypersensitive site at a position approximately −3.0 kb, designated as HSm (male-specific hypersensitive site) (−3780/−2203), also appeared with nuclei from the livers of male rats. HS1 contained *cis*-acting elements, GH-responsive element (GHRE) (−4213 to −4161), 2C12-I (−4095 to −4074) and 2C12-II (−4072 to −4045), which were essential for the GH-induced expression of the *CYP2C12* gene (29). The GHRE was recognized by the heterodimer of STAT5a with STAT5b. 2C12-I interacted with cAMP response element binding protein (CREB). 2C12-II bound to HNF-3α and CCAAT/enhancer-binding protein (C/EBP) α. The sequence of DNase I hypersensitive sites, HS2, HS3, and HSm, did not overlap with any binding sites previously identified by us (29). The expected size of fragment protected from DNase I was 1.7 kb, when genomic DNA from DNase I-treated nuclei was cleaved with *Sac*I (Fig. 3, C and D). In addition to the above hypersensitive sites, we found additional hypersensitive sites at positions −0.1 and −0.5 kb, named as HS4 and HS5, respectively. HS4 was specific for females, although HS5 was present in males and females. HS4 possessed binding sites for HNF-4 (−122 to −96), HNF-6 (−52 to −30), and TATA box recognized by basal transcription factors (Fig. 3D). HNF-4- and HNF-6-binding sites were also required for the GH-dependent activation of the *CYP2C12* gene (29). The sequence of HS5 did not overlap with any binding sites for known transcription factors. On the basis of these lines of evidence, it seemed that the female-specific expression of the *CYP2C12* gene results from the inaccessibility of STAT5, HNF-4, HNF-6 and basal transcription factors to their target elements due to chromatin condensation seen in male rat livers.

Functional Analysis of HSm

To examine whether or not the activity of simian virus 40 (SV40) promoter was affected by the presence of HSm, HSm was inserted in front of SV40 promoter (pHSm) (Fig. 4A). The DNA of pHSm reporter gene was injected into the liver of male rats. Luciferase activity seen with pHSm was one third of that with control vector. Thus, the result indicates that HSm functions as a negative regulatory

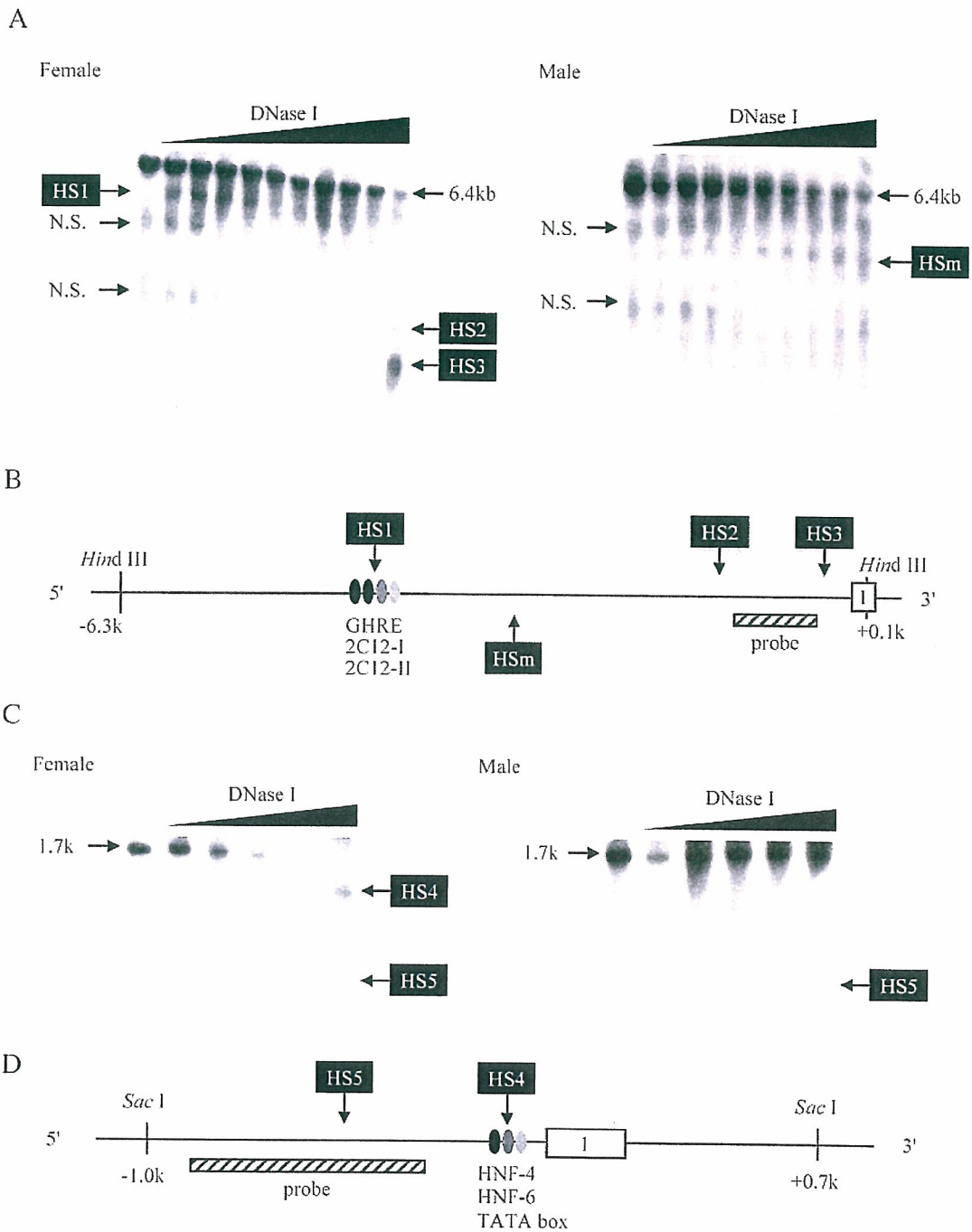

Fig. 3. Detection of DNase I Hypersensitive Sites in the Distal Enhancer and Proximal Promoter Regions of the *CYP2C12* Gene

A, Southern blot analysis demonstrating DNase I hypersensitive sites in the chromatin of rat livers. The nuclei were treated with DNase I (4–30 U/ml), followed by the isolation of genomic DNA. The genomic DNA was digested with *Hin*dIII, and then allocated to Southern blot analysis. Hypersensitive sites are shown by *arrows* (HS1–3, and HSm). N.S., Nonspecific band. B, Genomic organization of the *CYP2C12* gene. The region from nucleotides −927 to −359 of the *CYP2C12* gene was used as a probe, indicated by *hatched box*. *Open box* shows exon 1. C, Southern blot analysis demonstrating DNase I hypersensitive sites in the proximal promoter. The nuclei were treated with DNase I (4–25 U/ml), followed by the isolation of genomic DNA. The genomic DNA was digested with *Sac*I, and then allocated to Southern blot analysis. Hypersensitive sites (HS4 and HS5) are shown by *arrows*. D, Genomic organization of the *CYP2C12* gene. The region from nucleotides −927 to −359 of the *CYP2C12* gene was used as a probe, indicated by *hatched box*. *Open box*, Exon 1.

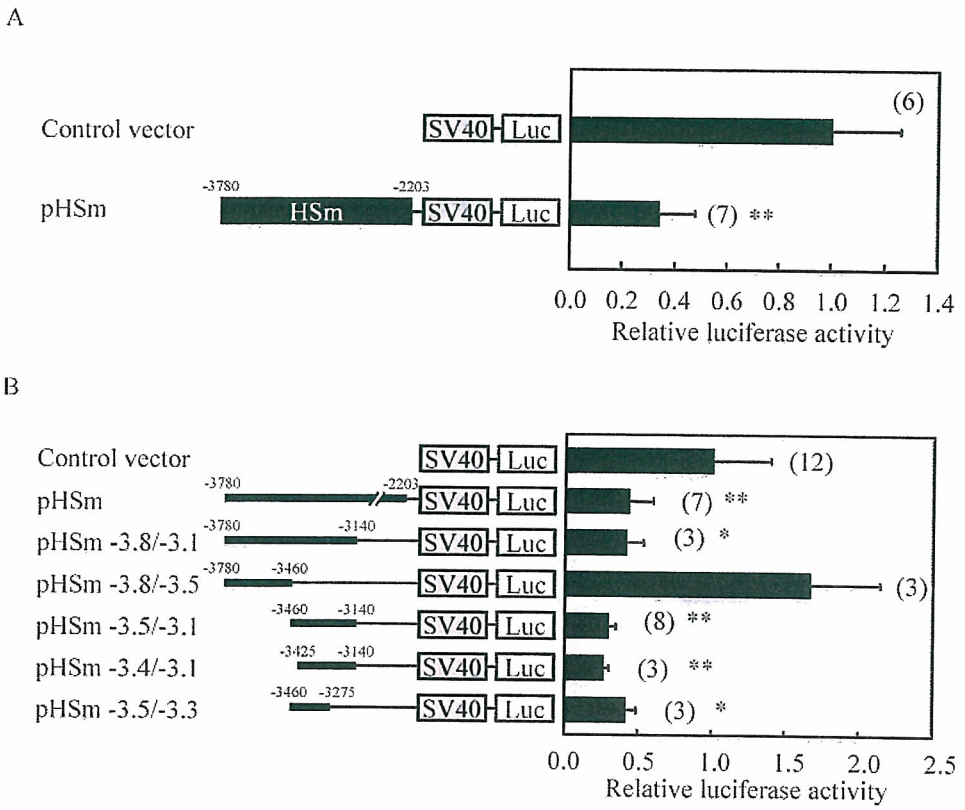

Fig. 4. Identification of the Negative Regulatory Region in the HSm of the *CYP2C12* Gene

A, Luciferase assay in the liver treated with pHSm. A reporter plasmid pHSm was injected into the livers of male rats. B, Luciferase assay with reporter plasmids with mutations in the HSm. The construction of deletion mutants is described in *Materials and Methods*. All values represent the mean ± SD from independent experiments shown in *parentheses*. The data are expressed as the ratio of the luciferase activity of each deletion mutant to the basal activity obtained with control vector. * and **, Significantly different from the activity in the liver transfected with the control vector at $P < 0.05$ and $P < 0.01$, respectively.

region. To identify one or more possible negative regulatory elements within HSm, we constructed reporter plasmids, pHSm-3.8/−3.1 (harboring a −3780/−3140 region), pHSm-3.8/−3.5 (−3780/−3460), pHSm-3.5/−3.1 (−3460/−3140), pHSm-3.4/−3.1 (−3425/−3140) and pHSm-3.5/−3.3 (−3460/−3275) (Fig. 4B). Luciferase activities with pHSm-3.8/−3.1, pHSm-3.5/−3.1, pHSm-3.4/−3.1 or pHSm-3.5/−3.3, was less than 50% of that with control vector. On the other hand, luciferase activity with pHSm-3.8/−3.5 increased 1.6-fold as compared with control vector. These results indicate that a region between nucleotides −3425 to −3275 had a possible negative regulatory element(s).

Characterization of Possible Negative Regulatory Elements

Searching the sequence similar to the −3425/3275 region within HSm, we found that the sequence of the region overlapped with those of putative binding sites for Octamer transcription factor-1 (Oct-1) and C/EBP (Fig. 5A). To examine the *in vivo* function of the binding sites for Oct-1 and C/EBP, we generated mutant reporter plasmids, pHSm mt(C/EBPsite1), pHSm mt(Oct-1, C/EBPsite1), pHSm mt (C/EBPsite2), pHSm mt(C/EBPsite3) and pHSm mt(Oct-1, C/EBPsite1, 2, 3) (Fig. 5B). Luciferase activity with pHSm mt(C/EBPsite3) was 3-fold higher than that with pHSm −3.5/−3.1, and was comparable with that with control vector. Introduction of mutations into Oct-1, C/EBPsite1, C/EBPsite2, and C/EBPsite3 [pHSm mt(Oct-1, C/EBPsite1, 2, 3)] resulted in a 1.4-fold increase in luciferase activity compared with control vector. However, the luciferase activities with pHSm mt(C/EBPsite), pHSm mt(Oct-1, C/EBPsite1), pHSm mt(C/EBPsite2) did not reach the level of luciferase activity with control vector. Thus, C/EBPsite3 seemed to be a negative regulatory element in male rat livers.

To clarify one or more factors to bind to the C/EBPsite3, supershift shift assay using nuclear extracts from male rat livers and antibodies to C/EBPα or C/EBPβ was conducted (Fig. 5C). The addition of antibodies to C/EBPα supershifted complexes I, II, and V, whereas the addition of antibodies to C/EBPβ supershifted complexes I, III, and IV. Thus, we concluded that C/EBPα and/or C/EBPβ bound to the C/EBPsite3 in male rats.

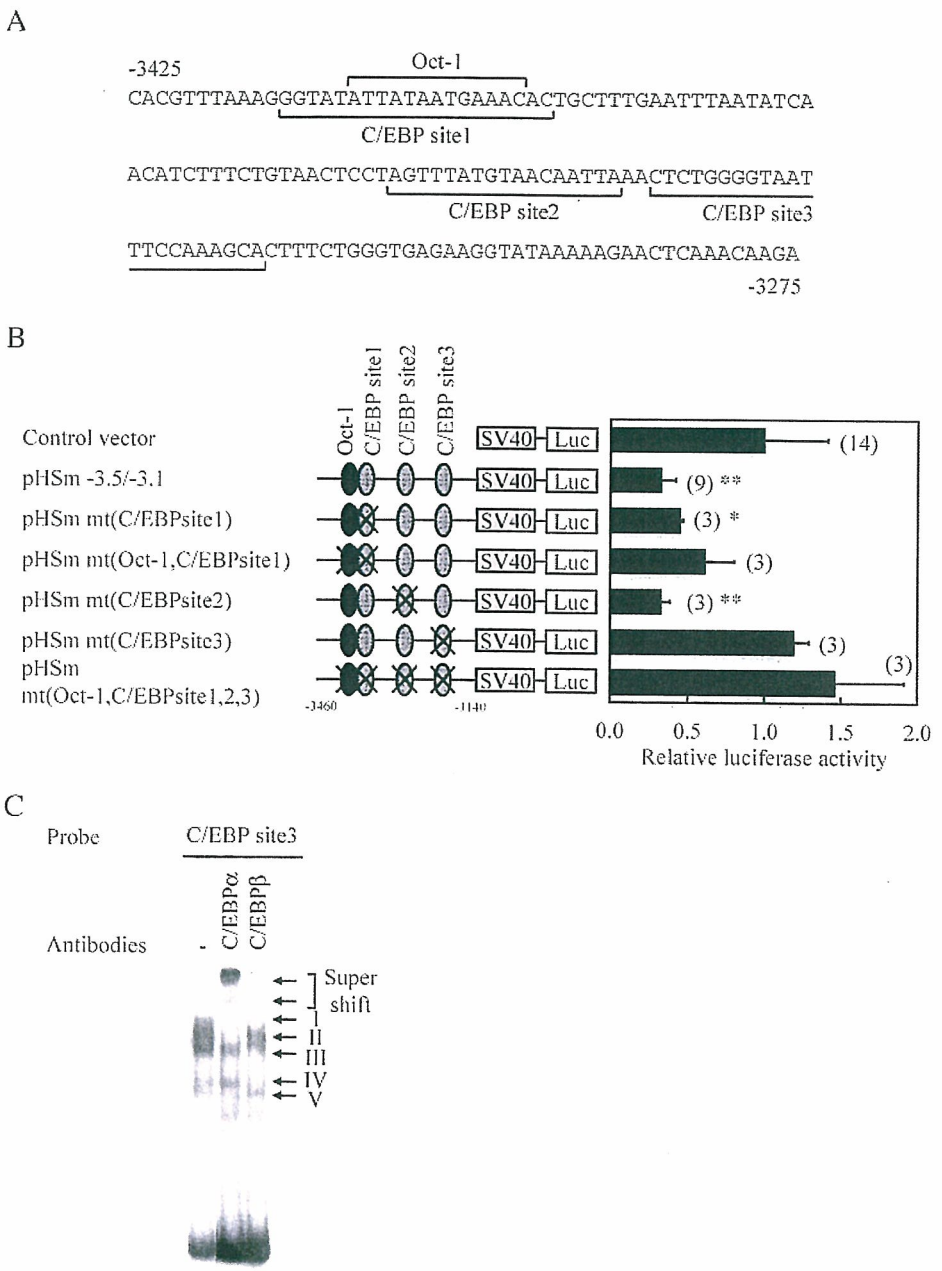

Fig. 5. Binding of C/EBP to the Negative Regulatory Element in the HSm of the *CYP2C12* Gene

A, Nucleotide sequences from −3425 to −3275 of the *CYP2C12* gene. Putative Oct-1 and C/EBP binding sites are indicated by *brackets*. B, Functional roles of putative binding sites for Oct-1 and C/EBP in a transcriptional repression activity. The construction of mutant plasmids is described in *Materials and Methods*. The mutants were injected into the livers of male rats. All values represent the mean ± SD. from independent experiments shown in *parentheses*. The luciferase activity of different constructs is shown relative to that observed with the control vector. Each luciferase activity is normalized with the activity of Renilla luciferase coinjected as pRL-SV40. * and **. Significantly different from the activity in the liver transfected with the control vector at $P < 0.05$ and $P < 0.01$, respectively. C, A supershift assay with antibodies to C/EBPα, or C/EBPβ. ^{32}P-Labeled C/EBPsite3 was incubated with the nuclear extracts from the liver of male rats in the presence or absence of antibodies (1 μl) to C/EBPα, or C/EBPβ. Supershifted bands are shown by *arrows*.

DISCUSSION

A proposed model for the gender-related expression of the *CYP2C12* gene is shown in Fig. 6. The gender-related expression of the *CYP2C12* gene can be accounted for by the inaccessibility of STAT5 HNF-4, HNF-6, and basal transcription factors to their target elements due to chromatin condensation

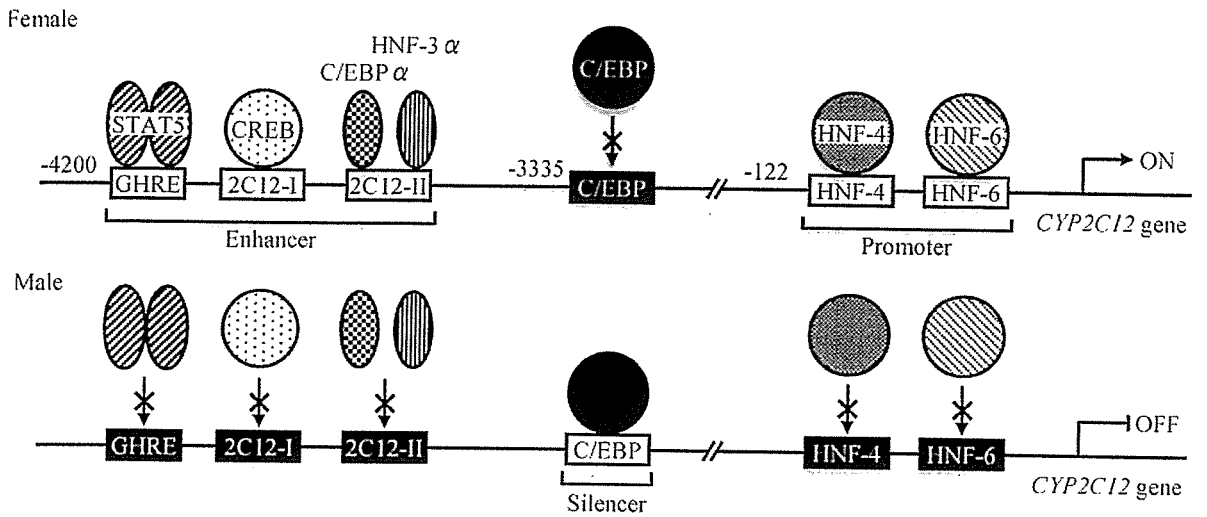

Fig. 6. Proposed Model for the Gender-Related Transcriptional Regulation of the *CYP2C12* Gene
White boxes, Regions in the open chromatin structure: *Black boxes*, Regions in the closed chromatin conformation.

seen in male rat livers but not in female rat livers, and by the presence of C/EBPsite3, a negative regulatory element(s) in the male-specific DNase I hypersensitive site.

By using direct DNA injection, we could not confirm the gender-related expression of the *CYP2C12* gene in rats. Because a naked plasmid was transfected into the livers, sex difference in chromatin structure cannot be seen in the present experiments.

CYP2C12 is unique in its female-specific expression. On the other hand, STAT5 is considered to be a male-enriched transcriptional factor, although we have shown that STAT5 serves as the modulator of *CYP2C12* gene expression in female as well as male rats. Actually, the activation of the Luc10kb reporter gene was also seen in male rats as well as female rats. This result does not agree with the previous finding that STAT5b inhibits the transcriptional activation of the *CYP2C12* gene by HNF-6 in HepG2 cells (30). In our experiments, a reporter plasmid Luc10kb with native GHRE was used for direct DNA injection. On the other hand, in the experiments performed by Waxman's group (30), luciferase reporter constructs used for DNA transfection were without native GHRE. Thus, the inhibition of *CYP2C12* gene expression by STAT5b might not be seen *in vivo*.

A gender-related difference has also been reported in the expression level of HNF-6 (27). HNF-6 functioned as the activator of the Luc10kb reporter gene in male rats. Thus, HNF-6 may not be a critical factor determining the gender-related difference.

To avoid the possibility that the gender-related expression of the *CYP2C12* gene is caused by a difference in the amount of *trans*-acting factors, STAT5, CREB, HNF, and C/EBP in the liver, a gel shift assay using *cis*-acting elements, GHRE, 2C12-I, 2C12-II, HNF-4, and HNF-6 as probes and nuclear extracts from the livers of male or female rats was carried out (data not shown). However, the gender-related expression of the *CYP2C12* gene did not correlate with the amounts of binding of these *trans*-acting factors to the target elements.

In our previous study, we found that the 2C12-I bound to CREB, known to be a transcription factor that interacts with p300/CBP (CREB-binding protein) that functions as histone acetyltransferase (32). In addition to CREB, STAT5 also interacts directly with p300/CBP (33). Therefore, STAT5 may function synergistically with CREB through p300/CBP to activate chromatin structure.

Our site-directed mutagenesis and gel shift experiments suggest that C/EBPα and/or C/EBPβ act as a repressor. Interestingly, the region containing C/EBPsite3 was accessible by DNase I in the male but not female livers. Recently, chromatin immunoprecipitation technique, which provides us with direct information about interactions between the *trans*-acting factors and the *cis*-acting element of interest, has been developed (34). To further resolve the repressor complex interacting with C/EBPsite3, currently we are planning the chromatin immunoprecipitation assay.

It has been reported that C/EBPα and C/EBPβ interact with the complex of SWI (switching defective) with sucrose nonfermenting (SNF), known to participate in chromatin remodeling (35, 36). Recently, a major subunit Brahma-related gene (Brg1) homologous to SWI2 has been reported to be contained in a component including corepressor nuclear receptor corepressor and HDAC (37). Thus, SWI/SNF is involved in the negative regulation of target genes (38–40). SWI/SNF complex may be recruited by C/EBP on the *CYP2C12* gene, and then inactivates the chromatin structure of the *CYP2C12* gene in male rats. Alternatively, the modifications of C/EBP protein such as SUMOylation may be involved in the gender-specific expression of the *CYP2C12* gene (41–43)

One or more cofactors that responds to GH continuously secreted in female rats may also be required because treatment of male rats with TSA alone did not increase the expression level of CYP2C12 mRNA. These factors are currently under examination.

In the present study, we propose a novel mechanism to explain the gender-related expression of the *CYP2C12* gene in rats, which was completely distinct from the mechanism by which STAT5b inhibits the transcription of the *CYP2C12* gene by HNF-6 in HepG2 cells (30).

MATERIALS AND METHODS

Animal Treatments

Adult male or female Harlan Sprague Dawley rats (7 wk old; Sankyo Experimental Animals, Tokyo, Japan) were used. When necessary, male and female rats were hypophysectomized at 6 wk of age. TSA (Sigma, St. Louis, MO) was administered by a continuous infusion (0.1 mg/kg·d) with an osmotic minipump (model 2001, Alza, Palo Alto, CA) for 24 h. Recombinant human methionylated GH (Somatonorm, Kabi Vitrum, Stockholm, Sweden) was kindly supplied by Sumitomo Pharmaceutical Co. (Osaka, Japan). After the treatment with TSA, human GH was administered by a continuous infusion (0.94 IU/kg·d), which mimics female-type GH secretion, with an osmotic minipump for 7 d.

Construction of Reporter Plasmids for Luciferase Assay

A reporter plasmid Luc10kb was constructed as follows. First, a clone containing the 5′-flanking region up to nucleotides −10 kb of the *CYP2C12* gene (29) was digested with restriction enzymes, *Xba*I and *Xho*I. Resultant fragment was inserted into the *Nhe*I/*Xho*I site of Luc5132 (29). To construct a reporter plasmid pHSm −3.8/−3.1, Luc4200 (29) was digested with *Bgl*II/*Hin*cII. Resultant fragments were blunt-ended with T_4 DNA polymerase (Takara, Osaka, Japan). Then these fragments were inserted into the *Sma*I site of the PicaGene Control Vector 2 (Toyolnk, Tokyo, Japan). The direction of the inserts was confirmed by a sequence analysis (ABI PRISM 377; PerkinElmer Life Sciences, Foster City, CA). Reporter plasmids, pHSm −3.8/−3.5, pHSm −3.5/−3.1, pHSm −3.4/−3.1, and pHSm −3.5/−3.3, were generated by PCR using S-3780, S-3460, or S-3425 as a 5′-primer and AS-3460, AS-3140, or AS-3275 as a 3′-primer, respectively. The sequence of these primers is shown below. Synthesized fragments were digested with *Mlu*I/*Xho*I. Resultant fragments were inserted into the *Mlu*I/*Xho*I site of the Control Vector 2. To examine the function of putative DNA elements, Oct-1, C/EBPsite1, C/EBPsite2, C/EBPsite3, these DNA elements were mutated as follows. Mutant reporter plasmids, pHSm mt(C/EBPsite1), pHSm mt(Oct-1, C/EBPsite1), pHSm mt(C/EBPsite2), pHSm mt(C/EBPsite3), and pHSm mt(Oct-1, C/EBPsite1, 2, 3) were produced by site-directed mutagenesis using S-mtC/EBPsite1, AS-mtC/EBPsite1, S-mtOct-1,C/EBPsite1, AS-mtOct-1,C/EBPsite1, S-mtC/EBPsite2, AS-mtC/EBPsite2, S-mtC/EBPsite3, or AS-mtC/EBPsite3 as mutated primers. A reporter plasmid Luc4200 mt(C/EBPsite3) was constructed by ligation of the following three DNA fragments: 1) a *Kpn*I/*Dra*I fragment (−4200/−3420) from Luc4200; 2) a *Dra*I/*Eco*RV fragment (−3420/−3140) from pHSm mt(C/EBPsite3); 3) a 7926 bp *Kpn*I/*Eco*RV fragment from Luc4200. The oligonucleotide primers used for the synthesis of DNA fragments or for a site-directed mutagenesis are as follows. The mutated nucleotide sequences are *underlined*.

S-3780, 5′-TTTACGCGTAGATCTCTGTA-3′; S-3460, 5′-GATACGCGTTATGATAAGGC-3′; AS-3460, 5′-CTTCTCGAGGTACACATCTG-3′; S-3425, 5′-TTTACGCGTCACGTTTAAAG-3′; AS-3275, 5′-AACCTCGAGTCTTGTTTGAG-3′; AS-3140, 5′-AAACTCGAGGATATCCTTGT-3′; S-mtC/EBPsite1, 5′-CACGTTTAAAGGGTATATTATAATGAGGCACTGCTT-3′; AS-mtC/EBPsite1, 5′-AAGCAGTGCCTCATTATAATATACCCTTTAAACGTG-3′; S-mtOct-1, C/EBPsite1, 5′-CACGTTTAAAGGGTATATTATCCTGAGGCACTGCTT-3′; AS-mtOct-1, C/EBPsite1, 5′-AAGCAGTGCCTCAGGATAATATACCCTTTAAACGTG-3′; S-mtC/EBPsite2, 5′-ACTCCTAGTTTATGT GCCGGTTAAACTCTGGGGTA-3′; AS-mtC/EBPsite2, 5′-TACCCCAGAGTTTAACCGGCACATAAACTAGGAGT-3′; S-mtC/EBPsite3, 5′-TAAACCCTGGGGTCGTTTCCGCGGCACTTT-3′; AS-mtC/EBPsite3, 5′-AAAGTGCCGCGGAAACGACCCCAGGGTTTA-3′.

Direct DNA Injection and Luciferase Assay

Direct DNA injection and luciferase assay were performed as previously described (29). When a reporter plasmid with SV40 promoter was used, 4 μg of a pRL-SV40 (Promega, Madison, WI) was coinjected into the liver of rats as an internal control. Rats were killed 1 d after the injection, when the highest luciferase activity was seen. Statistical analysis was performed by a Mann-Whitney *U* test with a commercially available statistical package (SPSS version 11.0; SPSS Japan Inc., Tokyo, Japan).

Northern Blot Analysis

Total RNAs were prepared from the livers of male and female rats according to the method of Chomczynski *et al.* (44). An oligonucleotide specific for *CYP2C12* (5′-AATAGCAGCAAAATGTTTTGAATGTGTCTT-3′) (45) was used as a probe. Total RNA (20 μg) was electrophoresed in a 0.8% agarose gel containing 18% formaldehyde and was transferred to a nylon membrane (Nytran NY13, Schleicher & Schuell, Dassel, Germany). The membrane was hybridized with ^{32}P-labeled oligonucleotide by [γ-^{32}P] ATP (Amersham Pharmacia Biotech, Tokyo, Japan) and T_4 polynucleotide kinase (Takara, Osaka). Hybridization was carried out by the method of Sambrrok *et al.* (46). The membrane was washed twice with 0.1 M sodium phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate, and 1 mM EDTA at 42 C for 30 min. The same membrane was used to determine the expression level of β-actin mRNA.

Preparation of Rat Liver Nuclear Extracts

Nuclear extracts were prepared from the livers of male and female rats as previously described (29).

Gel Shift Assay

The gel shift assay was performed as previously described (29). Antibodies to C/EBPα, and C/EBPβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The oligonucleotide primers used as a probe are as follows: C/EBPsite3, 5′-AATTAAACTCTGGGGTAATTTCCAAAGCACTTT-3′ and 3′-CAGAAAGTGCTTTGGAAATTACCCCAGAGTTTA-5′.

DNase I Hypersensitive Assay

DNase I hypersensitive assay was conducted essentially according to the method of Parnaik *et al.* (47) with minor modifications. Briefly, nuclei were prepared from the livers of male and female rats. The nuclei were treated with DNase I (4–30 U/ml), followed by the isolation of genomic DNA. The

genomic DNA was digested with *Hind* III or *Sac*I, and then allocated to Southern blot analysis. A region from nucleotides −927 to −359 of the *CYP2C12* gene was used as a probe.

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Inhibitory Effects of Nicardipine to Cytochrome P450 (CYP) in Human Liver Microsomes

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To anticipate drug–drug interactions by nicardipine *in vivo*, cytochrome P450 (CYP) forms responsible for the metabolism of nicardipine and inhibition of CYP-dependent drug metabolism by nicardipine were investigated. Microsomes of human B-lymphoblastoid cells expressing each human CYP form were used for the metabolism of nicardipine. Inhibitory effects of nicardipine on drug metabolism were studied using human liver microsomes. CYP2C8, CYP2D6 and CYP3A4 were identified as major CYP forms for the metabolism of nicardipine in human liver microsomes. Nicardipine strongly inhibited two-pathways of triazolam hydroxylation both catalyzed by CYP3A4. Comparison of three Ca^{2+} antagonists, nicardipine, nifedipine, and diltiazem revealed that only nicardipine showed such a strong inhibitory potency on the typical CYP2D6-catalyzed drug metabolism. Furthermore, nicardipine inhibited other reactions catalyzed by CYP1A, CYP2A6, CYP2C8, CYP2C9 and CYP2C19 with K_i values ranging from 1.1 to 29.4 μM. In conclusion, nicardipine was a relatively potent inhibitor of human CYP2D6, CYP3A4 and CYP2C (especially for CYP2C8 and CYP2C19) *in vitro*, suggesting that drug–drug interactions between nicardipine and other drugs metabolized mainly by these CYP forms appear to occur *in vivo*.

Key words Ca^{2+} blocker; diltiazem; drug–drug interaction; nifedipine

Nicardipine, a dihydropyridine derivative of calcium entry blockers, is widely used in the treatment of cardiovascular diseases. Clinical use of this drug has been approved for the treatment of hypertension.[6)] Many clinically used drugs are metabolized to pharmacologically inactive forms by one or more cytochrome P450s (CYPs).[7,8)] Recently, particular efforts have been paid to elucidating the pharmacokinetic mechanisms of drug–drug interactions. As one such mechanism, an administration of a certain drug alters the metabolism of other drugs *via* modulation of CYP activities. This interaction occurs by at least two distinct routes: induction and inhibition of CYP. In the latter case, a direct interaction of a drug with a certain CYP results in inhibition of its catalytic activity toward other drugs in a competitive or noncompetitive manner, or enhancement of its activity through allosteric activation. If a drug possessing potent inhibitory effects on CYP is administered simultaneously with another drug having a narrow therapeutic index, an exaggerated response and/or serious toxic effects may occur even at therapeutic doses as a result of increased plasma concentration of the parental drug. For instance, quinidine treatment inhibits CYP2D6 activity to cause orthostatic hypotension by increasing the plasma concentration of co-treated debrisoquine.[9)] In the same way, nicardipine was suspected as a modulator of other CYP substrates by information received from a patient. In the present study, we investigated the CYPs responsible for the metabolism of nicardipine in human liver microsomes. Then, inhibitory effects of nicardipine on reactions catalyzed by eight human CYP forms were examined to determine whether nicardipine would interact with other drugs in a CYP-mediated drug metabolism.

MATERIALS AND METHODS

Materials $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan), 7-ethoxycoumarin from Aldrich Chemical (Milwaukee, WI, U.S.A.), and nicardipine hydrochloride, nifedipine, diltiazem hydrocloride, coumarin, 7-hydroxycoumarin, *dl*-chlorpheniramine maleate and tolbutamide from Wako Pure Chemicals (Osaka, Japan). Sulfaphenazole was kindly provided by Ciba-Geigy Japan (Takarazuka, Japan), triazolam and triazolam metabolites from Pharmacia and Upjohn Japan (Tokyo, Japan), nicardipine metabolites from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan), midazolam and midazolam metabolites from Hoffmann-La Roche (Nutley, NJ, U.S.A.), and *S*-mephenytoin from Sumika Chemical Analysis Service (Tokyo, Japan), respectively. Pooled human liver microsomes were obtained from Daiichi Pure Chemicals (Tokyo, Japan). All other chemicals and solvents were of the highest grade commercially available.

Analytical Procedures Metabolites of nicardipine were determined as follows. A typical reaction mixture consisted of 100 mM potassium-phosphate buffer (pH 7.4), 50 μM EDTA, an NADPH-generating system (0.5 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate and 1 unit/ml glucose 6-phosphate dehydrogenase) and 0.2 mg protein of human liver microsomes in a final volume of 0.25 ml. After incubation at 37 °C for 10 min, ice-cold acetontrile (0.25 ml) was added to terminate the reaction. The mixture was cen-

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trifuged at 3000 rpm for 10 min and the resultant supernatant fraction was subjected to HPLC equipped with a L-7100 pump (Hitachi, Tokyo, Japan), a SPD-10A UV-visible absorbance detector (Shimadzu, Kyoto, Japan), a C-R6A integrator (Shimadzu, Kyoto, Japan) and an analytical column Nova-Pak Phenyl (3.9 mm×150 mm; Waters, Tokyo, Japan). The mobile phase consisting of 0.047 M ammonium acetate (pH 6.0)/acetonitrile (60 min of linear gradient from 81 : 19 to 54 : 46 v/v) was delivered at a flow rate of 0.8 ml/min. The elution of nicardipine and its metabolites was monitored at 238 nm. Coumarin 7-hydroxylase activity was measured as described by Pearce *et al.*[10] For this assay, 0.05 mg protein of microsomes was added to the reaction mixture and incubated at 37 °C for 5 min incubation. An F-2000 fluorescence detector (Hitachi, Tokyo, Japan) was employed for determination of a metabolite, 7-hydroxycoumarin. The elution of 7-hydroxycoumarin was monitored with excitation at 371 nm and emission at 454 nm. 7-Ethoxycoumarin *O*-deethylase activity was measured as described by Evans and Relling.[11] For this assay, microsomes (0.05 mg protein) were added and incubated for 30 min. HPLC equipped with a Capcell Pac C18 column (4.6 mm×250 mm, Shiseido, Tokyo, Japan) was employed for determination of a metabolite, 7-hydroxycoumarin. The elution of the parent compound and its *O*-deethylated metabolite was monitored with excitation at 360 nm and emission at 470 nm. *S*-Mephenytoin 4′-hydroxylase activity was determined as described by Yasumori *et al.*[12] The composition of an incubation mixture was the same as described above except that 0.1 mg protein of pooled microsomes was used. Incubations were performed at 37 °C for 60 min. Analysis of the metabolite was performed by HPLC equipped with a YMC-Pac Pro C18 column (4.6 mm×150 mm, YMC, Tokyo, Japan). The elution of *S*-mephenytoin and its 4′-hydroxylated metabolite was monitored at 204 nm. The method described by Cresteil *et al.*[13] was adopted for the assay of paclitaxel 6α-hydroxylase activity with minor modifications. Briefly, the final volume of the incubation mixture was 0.25 ml, and 0.1 mg protein of pooled microsomes was added to the reaction mixture. Incubations were carried out at 37 °C for 30 min. The same HPLC system employed for *S*-mephenytoin assay was used for the analysis of the 6α-hydroxy paclitaxel. The elution of paclitaxel and its 6α-hydroxylated metabolite was monitored at 230 nm. The assay of diclofenac 4′-hydroxylase activity was performed as described by Moncrieff[14] with minor modifications. Briefly, incubations were carried out at 37 °C for 15 min with 0.1 mg protein of pooled microsomes in a final incubation mixture of 0.25 ml. The metabolite was quantified by the same HPLC system as employed for the *S*-mephenytoin assay. The elution of diclofenac and 4′-hydroxylated metabolite was monitored at 280 nm. A typical incubation mixture for the assay of bufuralol and dextromethorphan metabolism[15] contained 100 mM potassium-phosphate (pH 7.4), 50 μM EDTA, substrate (bufuralol or dextromethorphan), the NADPH-generating system and 0.1 mg/ml of human liver microsomes in a final volume of 0.15 ml. To compare inhibitory potency on the metabolism of bufuralol with human liver microsomes, nicardipine, nifedipine or diltiazem was added to the incubation mixture. The reaction was stopped by addition of 60% perchloric acid. After centrifugation of the reaction mixture at 3000 rpm for 10 min, the supernatant was subjected to the HPLC systems equipped with a CCPE system pump (Tosoh, Tokyo, Japan), a C-R6A integrator (Shimadzu, Kyoto, Japan), an RF-530 Fluorescence HPLC monitor (Shimadzu, Kyoto, Japan) and an analytical column Capcell Pac C18 (4.6 mm×250 mm; Shiseido). The mobile phase consisting of 20 mM sodium perchlorate (pH 2.5)/acetonitrile (70 : 30, v/v) was delivered at a flow rate of 1.0 ml/min. The elution of the metabolites of bufuralol and dextromrthorphan was monitored fluorometrically with excitation at 252 nm and emission at 302 nm and with excitation at 270 nm and emission at 312 nm, respectively. Midazolam and triazolam hydroxylase activity was measured as described by Ring *et al.*[16] Briefly a typical incubation mixture for the assay of midazolam and triazolam metabolism contained 100 mM potassium-phosphate (pH 7.4), 50 μM EDTA, a substrate (midazolam or triazolam), the NADPH-generating system and 0.1 mg/ml of microsomal protein in a final volume of 0.25 ml. The sample was subjected to the same HPLC systems as described above, except that an analytical column TSK-GEL ODS-120T (4.6 mm×150 mm; Tosoh, Tokyo, Japan) was used. The mobile phase consisting of 100 mM sodium acetate (pH 7.8)/acetonitrile/methanol (53 : 29 : 18, v/v/v, for midazolam) or (61 : 24 : 15, v/v/v, for triazolam) was delivered at a flow rate of 1.0 ml/min. The elution of substrate and its metabolites was monitored at 240 nm.

Metabolism of Nicardipine by Human CYPs Expressed in Human B-Lymphoblastoid Cells Microsomes prepared from human B-lymphoblastoid cells expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9-Arg, CYP2C9-Cys, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 with human NADPH-CYP reductase were obtained from Daiichi Pure Chemicals (Tokyo, Japan). As a negative control, the microsomes of cells transformed with an expression plasmid without the CYP cDNA were used. Incubations were carried out as described above, except that 1 unit of human P450 reductase and microsomal protein (0.15 mg) were added in a final volume of 0.15 ml.

RESULTS

Metabolism of Nicardipine The proposed *in vitro* metabolic pathways of nicardipine in human liver microsomes[17] are illustrated in Fig. 1. Incubation of the pooled human liver microsomes with nicardipine yielded debenzylated and oxidized metabolites. The rates of nicardipine debenzylation

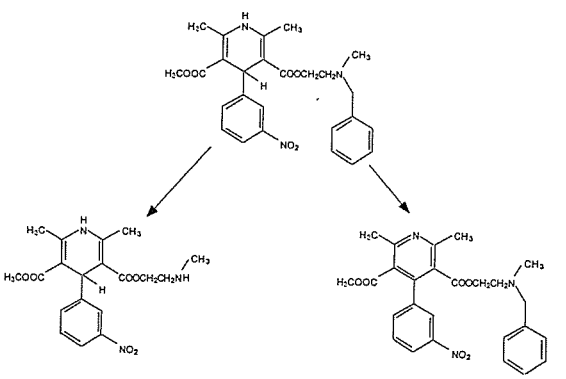

Fig. 1. Proposed Metabolic Pathways of Nicardipine in Human Liver Microsomes