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Constitutive Expression of Cytochrome P450 Genes in Newly Established Rat Hepatic Cell Lines

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Two cultured cell lines, called Kan-R1 and Kan-R2, were established from rat hepatic cells by *in vitro* culture with a hepatocarcinogen, 3-methoxy-4-aminoazobenzene, and examined for the gene expression of cytochrome P450 (P450) isoforms, CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and CYP3A2, by the RT-PCR method. It was revealed that all the P450 genes examined were expressed in both cell lines, although the two cell lines differed in cell size and colony-forming ability on a soft agar. The expression levels of the *CYP1A2, CYP2B1, CYP2B2, CYP3A1, and CYP3A2* genes were lower than those in liver tissues, while that of *CYP1A1* was higher in the cell lines. In both cell lines, cycloheximide, an inhibitor of protein synthesis, augmented the gene expression of the P450s except CYP2B1. These findings indicate that the newly established hepatic cell lines substantially express the P450 genes for CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1, and CYP3A2, and that the constitutive gene expression of these P450s, with the exception of CYP2B1, may be inhibited by negative transcription factors.

Key words: cycloheximide, CYP, cytochrome P450, gene regulation, hepatic cell lines.

Cytochrome P450 (P450) isoforms, comprising an important family of genetically controlled enzymes responsible for the metabolism of endogenous and exogenous chemicals, are present in various organs, especially the liver, and show different substrate specificities (*1*, *2*). The P450 isoforms responsible for the metabolism of xenobiotics such as carcinogens and drugs are generally induced by the xenobiotics themselves (*3*, *4*). Moreover, there are the species, sex, and organ differences in the constitutive and xenobiotic-induced levels of P450 isoforms (*5–8*).

The P450 isoforms are suggested to act as host factors that determine the biological susceptibility of individual animals and their organs to xenobiotics. For example, the susceptibility of rats and mice to hepatocarcinogenic aromatic amines, such as 3-methoxy-4-aminoazobenzene (*6*) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole acetate (*5*, *7*), is closely correlated with the constitutive and/or carcinogen-induced levels of carcinogen activation enzymes, particularly CYP1A2, in a target organ. Therefore, to understand these species, sex and organ differences, it is important to clarify the differences in cellular factor(s) responsible for the expression of each P450 isoform in animals and their organs.

For this purpose, it is necessary to determine the mechanism of expression of each P450 gene in an organ, but, except in the case of the *CYP1A1* gene, the mechanism remains unclear. To determine this mechanism, the establishment of an *in vitro* model may be valuable. However, the development of a cultured cell line expressing the genes of constitutive and/or xenobiotic-induced P450 isoforms has been unsuccessful, although it has been reported that some hepatoma cell lines (*9–13*) express the *CYP1A1* and *CYP1B1* genes.

In the present study, we established two cultured rat hepatic cell lines that express the genes of several P450 isoforms, and with the use of these cell lines we found that a negative transcription factor plays an important role in the constitutive gene expression of P450 isoform, *i.e.* CYP1A2, CYP2B2, CYP3A1 and CYP3A2.

MATERIALS AND METHODS

Chemicals—Cycloheximide was purchased from Sigma-Aldrich Japan, Tokyo. 3-Methoxy-4-aminoazobenzene (3-MeO-AAB) was synthesized in our laboratory by the method of Miller and Miller (*14*).

Establishment of Cultured Hepatic Cell Lines—Male Sprague-Dawley rats were obtained from Japan SLC Animals, Hamamatsu, and used at 7 weeks of age. They were kept in an air-conditioned room, and given CE-2 diet (CLEA Japan, Tokyo) and water *ad libitum* until used. A hepatocyte fraction was prepared by collagenase-perfusion according to the method of Williams (*15*). Isolated hepatic cells were suspended in Eagles' minimum essential medium containing 10% fetal calf serum (FCS) (MEM medium), and examined for viability by means of the trypan blue dye-exclusion test. A cell suspension containing more than 90% of viable hepatocytes was used for the establishment of the cultured rat hepatic cell lines.

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Table 1. **The PCR conditions used in the present experiments.**

Target gene	Primer set	Reaction conditions			Product size (bp)	Ref. No.
		denaturation	annealing	elongation		
Albumin	5′-TTGCCAAGTACATGTGTGAG-3′ (forward) 5′-GGTTCTTCTACAAGAGGCTG-3′ (reverse)	94°C, 60 sec	65°C, 60 sec	72°C, 60 sec	373	16
HNF-1	5′-AGGGCGGACTGATTGAAGAG-3′ (forward) 5′-CACCTCAGGCTTGTGGCTGTACAG-3′ (reverse)	95°C, 45 sec	59°C, 45 sec	72°C, 60 sec	979	17
HNF-4	5′-GCCTGCCTCAAAGCCATCAT-3′ (forward) 5′-GACCCTCCAAGCAGCATCTC-3′ (reverse)	95°C, 45 sec	59°C, 45 sec	72°C, 60 sec	285	17
C/EBP-α	5′-CCCGTGCCCAGCCCTCAT-3′ (forward) 5′-CACCTTCTGCTGCGTCTCCAC-3′ (reverse)	95°C, 45 sec	59°C, 45 sec	72°C, 60 sec	264	17
CYP1A1	5′-TGACCTCTTTGGAGCT-3′ (forward) 5′-TTGAGCCTCAGCAGAT-3′ (reverse)	95°C, 60 sec	50°C, 30 sec	72°C, 120 sec	1050	18
CYP1A2	5′-GATGAGAAGCAGTGGAAAGACC-3′ (forward) 5′-AAAAAGAAAGGAGGAACAA-3′ (reverse)	95°C, 60 sec	50°C, 30 sec	72°C, 120 sec	328	17
CYP2B1	5′-GCTCAAGTACCCCCATGTCG-3′ (forward) 5′-ATCAGTGTATGGCATTTTACTGCGG-3′ (reverse)	93°C, 60 sec	54°C, 90 sec	72°C, 60 sec	109	19
CYP2B2	5′-CTTTGCTGGCACTGAGACCG-3′ (forward) 5′-ATCAGTGTATGGCATTTTGGTACGA-3′ (reverse)	93°C, 60 sec	54°C, 90 sec	72°C, 60 sec	163	19
CYP2E1	5′-TCTGAGGCTCATGAGT-3′ (forward) 5′-GACAGGAGCAGAAACA-3′ (reverse)	95°C, 60 sec	50°C, 30 sec	72°C, 120 sec	770	18
CYP3A1	5′-GATGTTGAAATCAATGGTGTGT-3′ (forward) 5′-TTCAGAGGTATCTGTGTTTCC-3′ (reverse)	95°C, 60 sec	60°C, 60 sec	72°C, 120 sec	289	20
CYP3A2	5′-AGTAGTGACGATTCCAACATAT-3′ (forward) 5′-TCAGAGGTATCTGTGTTTCCT-3′ (reverse)	95°C, 60 sec	60°C, 60 sec	72°C, 120 sec	252	20
AhR	5′-CCCCAATTCCCTTATGAGTGC-3′ (forward) 5′-GGAGGAGTCGGTTCGGAAGA-3′ (reverse	95°C, 60 sec	60°C, 60 sec	72°C, 120 sec	340	21
CAR	5′-TCTCACTCAACACTACGTTC-3′ (forward) 5′-CTGGGAAAGGATCCACGCCTGGG-3′ (reverse)	95°C, 30 sec	58°C, 30 sec	72°C, 30 sec	450	22
RXR-α	5′-CCATGGCGTCCTCAAGGTTC-3′ (forward) 5′-ACTCCACCTCGTTCTCATTC-3′ (reverse)	95°C, 15 sec	55°C, 15 sec	72°C, 60 sec	326	23
PXR	5′-AGAGGCGGGCCTTGATCAAG-3′ (forward) 5′-GCATCAGCACATACTCCTCC-3′ (reverse)	94°C, 30 sec	58°C, 30 sec	72°C, 30 sec	672	-*
CYP51	5′-CATACAAGGATGGGCGTCC-3′ (forward) 5′-ATAAACGCCGCTAGTGGACC-3′ (reverse)	94°C, 30 sec	55°C, 30 sec	72°C, 120 sec	543	24
GAPDH	5′-TTCAACGGCACAGTCAAGG-3′ (forward) 5′-CATGGACTGTGGTCATGAG-3′ (reverse)	95°C, 60 sec	60°C, 60 sec	72°C, 120 sec	373	25

*The forward and reverse sequences selected as primers for rat PXR cDNA (Genebank locus No. AF151377) were localized at 567–586 and 1218–1238, respectively, in the cDNA sequence.

Isolated fresh hepatic cells were suspended in MEM medium, and aliquots (1.4×10^5 cells/500 μl) of the cell suspension were inoculated into a 48-well culture dish (Costar 3548). After preincubation for 1 h at 37°C in a humidified CO_2 incubator, the non-adherent cells were removed from the cultures, and the remaining adherent cells were further incubated for 24 h in fresh MEM medium. Thereafter, the medium was replaced with MEM medium containing 3-MeO-AAB (final concentration: 10^{-5}, 10^{-6}, or 10^{-7} M), the medium being renewed every 3 days. After the 60-day culture, proliferous cells appeared from the cells cultured in the medium containing 3-MeO-AAB (10^{-6} M), but not in the other media. These cells were harvested by treatment with 0.0075% actinase E-0.1% EDTA and cloned by the 1/2 limited dilution method. The resultant two cloned cell lines, called Kan-R1 and Kan-R2, which grew without pileup in the MEM medium in the absence of 3-MeO-AAB, were selected and used for studies on the gene expression of P450 isoforms.

RT-PCR Analyses of Liver-Selective/Enriched Proteins and P450 Isoforms—An aliquot (10^6 cells/6 ml) of a suspension of Kan-R1 or Kan-R2 cells in MEM medium was inoculated into a tissue culture dish (Costar 3038) and preincubated for 1 h at 37°C. After the preincubation, the non-adherent cells were removed from the cultures and the remaining adherent cells were further cultured for 24 h at 37°C in fresh MEM medium. After the culture, the medium was removed and the remaining adherent cells were dissolved with ISOGEN (NipponGene) for preparation of total RNA.

The RNA preparations were subjected to RT-PCR to determine the gene expression of liver-selective/enriched proteins, albumin, hepatocyte nuclear factors (HNF-1 and HNF-4), C/EBP-α, P450 isoforms (CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1, and CYP3A2), and transcription factors (arylhydrocarbon receptor, retinoid X receptor, constitutive androstane receptor, and pregnane X receptor), with GAPDH as an internal control. A portion (4 μg) of the total RNA was converted to cDNA using poly $d(N)_6$ primer (Pharmacia Biotech) and Moloney murine leukemia virus reverse transcriptase (GIBCO, BRL) in an RT-reaction mixture (20 μl). PCR was performed with 0.8 μl of the RT-reaction mixture, 0.5 μM of the corresponding primer set (Table 1), and AmpliTaq

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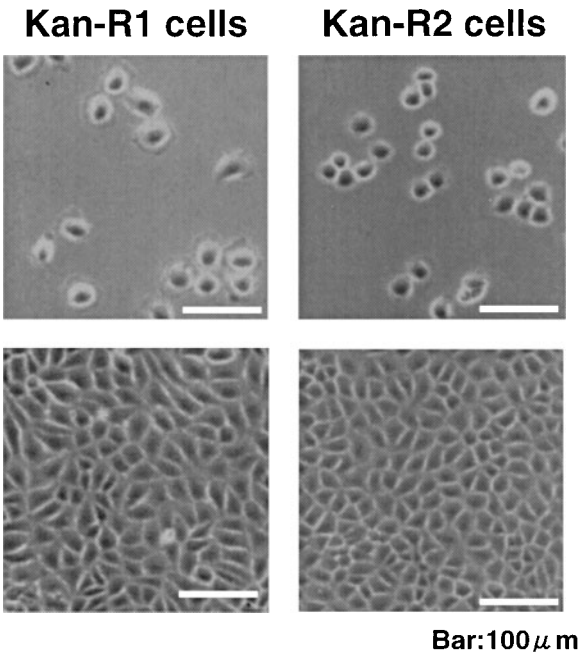

Fig. 1. **Morphology of the Kan-R1 and Kan-R2 cells.** Upper and lower photographs show the morphology of each established cultured cell line after 3 h-culture and 48 h-culture, respectively.

Gold DNA polymerase (PERKIN ELMER) in a total reaction volume of 25 μl. The amplification conditions are summarized in Table 1. PCR-products were separated by electrophoresis on a 2% agarose gel and then visualized under ultraviolet light after ethidium bromide staining. The product comprising each fluorescent band was densitometrically quantified using Kodak 1D Image Analysis Software (Machintosh 1D version 2.02).

The expression level of each P450 gene was normalized as to that of the *GAPDH* gene. In addition, to examine the contamination of DNA in the RNA preparation, each RNA preparation was subjected to PCR without RT reaction. The direct PCR gave no product, showing the presence of no contamination by DNA in the RNA preparations used.

RESULTS

Establishment of Hepatic Cell Lines—When primary cultured hepatic cells were incubated in MEM medium alone, they died within 7 days. On the other hand, a 60-day-culture with 3-MeO-AAB at a final concentration of 10^{-6} M, but not at 10^{-5} M or 10^{-7} M, gave proliferous cells. The resultant cells were cloned to afford two hepatic cell lines, called Kan-R1 and Kan-R2. They could grow even in the absence of 3-MeO-AAB. The morphology of Kan-R1 and Kan-R2 cells is shown in Fig. 1. Kan-R1 was larger than Kan-R2 in cell size. Both cell lines grew without pileup and formed a monolayer, and it was possible to store them by freezing in liquid nitrogen without loss of viability. Kan-R2 cells, but not Kan-R1 cells, have the capacity to form colonies on soft agar containing 10% FCS-DMEM medium (data not shown).

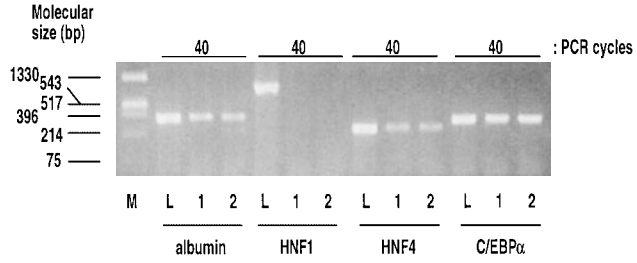

Fig. 2. **Gene expression of hepatocyte-selective/enriched proteins in the Kan-R1 and Kan-R2 cell lines.** Lanes M, L, 1 and 2: molecular markers, liver tissue, Kan-R1 and Kan-R2, respectively.

To determine whether or not the established cell lines had kept the characteristics of parenchymal cells, we examined the gene expression of albumin, hepatocyte nuclear factors (HNF-1 and HNF-4), and C/EBP-α, which are liver-selective/enriched proteins (*16*, *17*). Both cell lines were found to express the genes for albumin, HNF-4, and C/EBP-α, but not the gene for HNF-1, although the expression levels of these genes were much lower than those in liver tissues (Fig. 2).

Expression of P450 Genes—The gene expression of several P450 isoforms in Kan-R1 and Kan-R2 cells was examined by the RT-PCR method. In both cell lines, significant levels of expression of the *CYP1A1, CYP1A2,* and *CYP2B1* genes were observed, whereas those of the *CYP2B2, CYP3A1,* and *CYP3A2* genes were low (Fig. 3). In the Kan-R1 and Kan-R2 cell lines, the expression levels of the P450 genes, with the exception of the *CYP3A1* one, were almost the same, although that of the *CYP3A1* gene was slightly higher in Kan-R2 cells than in Kan-R1 cells.

The expression of P450 genes in the Kan-R1 and Kan-R2 cells showed a different pattern from that in liver tissues (Fig. 3). The expression level of the *CYP1A1* gene

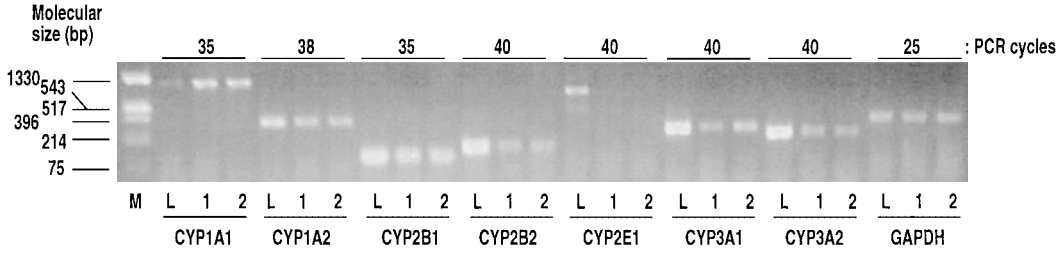

Fig. 3. **Gene expression of several P450 isoforms in the cultured hepatic cell lines, Kan-R1 and Kan-R2, and liver tissue.** Lanes M, L, 1 and 2: molecular markers, liver tissue, Kan-R1 and Kan-R2, respectively. The *GAPDH* gene was used as an internal standard.

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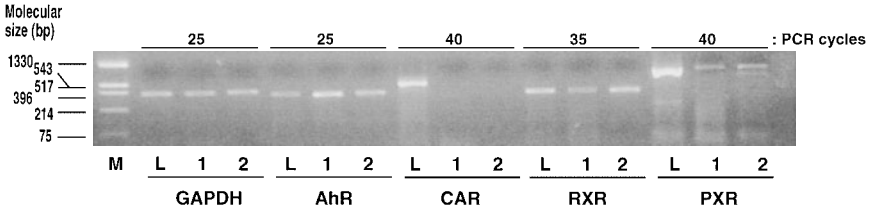

Fig. 4. **Gene expression of several transcription factors responsible for several P450 genes in the cultured hepatic cell lines, Kan-R1, Kan-R2, and the liver tissue.** Lanes M, L, 1 and 2: molecular markers, liver tissue, Kan-R1 and Kan-R2, respectively. The *GAPDH* gene was used as an internal standard.

was higher in the cultured cell lines than in the liver, while those of the other P450 genes were opposite. In addition, no expression of the *CYP2E1* gene was observed in the established cell lines. As to GAPDH, an internal standard, the gene expression levels in the cell lines and liver tissues were almost the same.

Transcription Factors Responsible forP450 Gene Expression—The gene expression levels of aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), retinoid X receptor (RXR), and pregnane X receptor (PXR) were examined in both Kan-R1 and Kan-R2 cell lines and compared with those in liver tissues (Fig. 4). The *AhR* and *RXR* genes were clearly expressed in both cell lines as well as in liver tissues. On the other hand, the *CAR* gene was only expressed in liver tissues, *i.e.* not in the cell lines. Expression of the PXR gene was clearly observed in liver tissues, but only slightly in the cell lines.

Effect of Cycloheximide (CHX) on Expression of the P450 Genes—The effect of CHX, an inhibitor of protein synthesis, on expression of the P450 genes was examined in the established cell lines, Kan-R1 and Kan-R2. Representative expression patterns of the P450 genes in Kan-R2 cells are depicted in Fig. 5. Culturing of Kan-R2 cells in the presence of CHX resulted in increases in the expression levels of the *CYP1A1, CYP1A2, CYP2B2,* and *CYP3A1* genes, but no clear change in expression of the *CYP2B1* gene was observed. The expression levels of the *CYP1A1* and *CYP3A2* genes gradually increased up to 48 h on culture with CHX (Fig. 6). The expression levels of the *CYP1A2* and *CYP3A1* genes were increased at 3 h, and further increases were observed up to 48 h. The gene expression level of CYP2B2 gradually increased until 24 h, and the increased level was slightly reduced at 48 h, whereas no clear change in the gene expression of CYP2B1 was observed until 48 h. By contrast, the gene expression of CYP51 (lanosterol 14α-demethylase) was decreased to about 50% of the control level at 12 h on culture with CHX, and the decreased level was maintained up to 48 h. No significant change in the gene expression of GAPDH, an internal standard, was observed until 48 h. In addition, similar changes in the expression of the P450 genes on culture with CHX were observed in Kan-R1 cells (data not shown).

DISCUSSION

In this work we established two cultured cell lines, Kan-R1 and Kan-R2, from rat primary cultured hepatic cells by means of long-term culture with 3-MeO-AAB, a hepatocarcinogen (*26*) and CYP1A2-selective inducer (*27*), and found that the cell lines still expressed the genes for CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and CYP3A2. The cell lines appear to be derived from hepatocytes, because the cells express the genes for hepatocyte-selective and/or enriched proteins such as albumin, HNF-4, and C/EBP-α. Although hepatic cell lines, Kan-R1 and Kan-R2, were here established with the use of 3-MeO-AAB, we failed to establish such cell lines with the use of 2-amino-6-methyl-dipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-

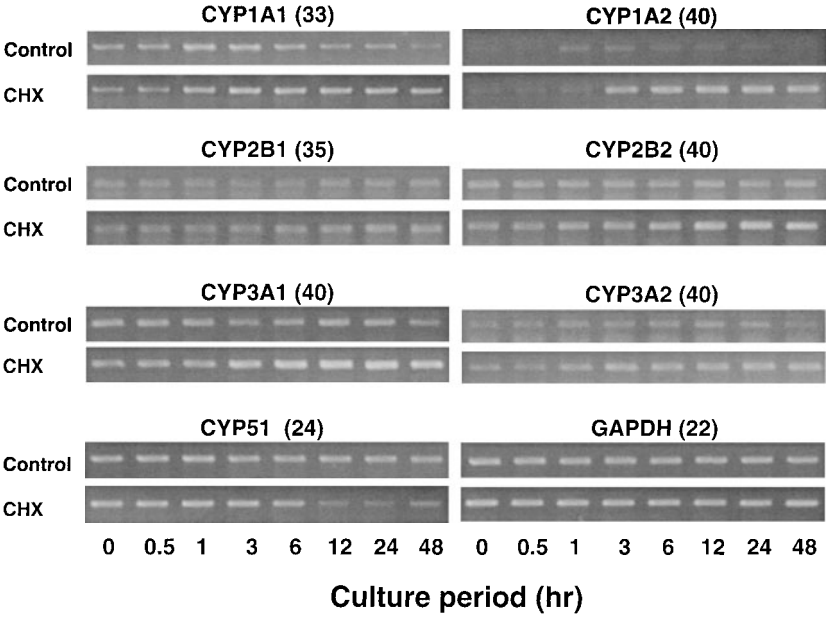

Fig. 5. **Representative expression patterns of the P450 genes in CHX-treated Kan-R2 cells.** Total RNAs were prepared from Kan-R2 cells cultured in the presence and absence of CHX (10 μg/ml) for the indicated times, and used for RT-PCR analysis of the expression of each P450 gene expression as described under "MATERIALS AND METHOD". Each culture medium was renewed at time 0. The values in parentheses represent the numbers of PCR cycles performed.

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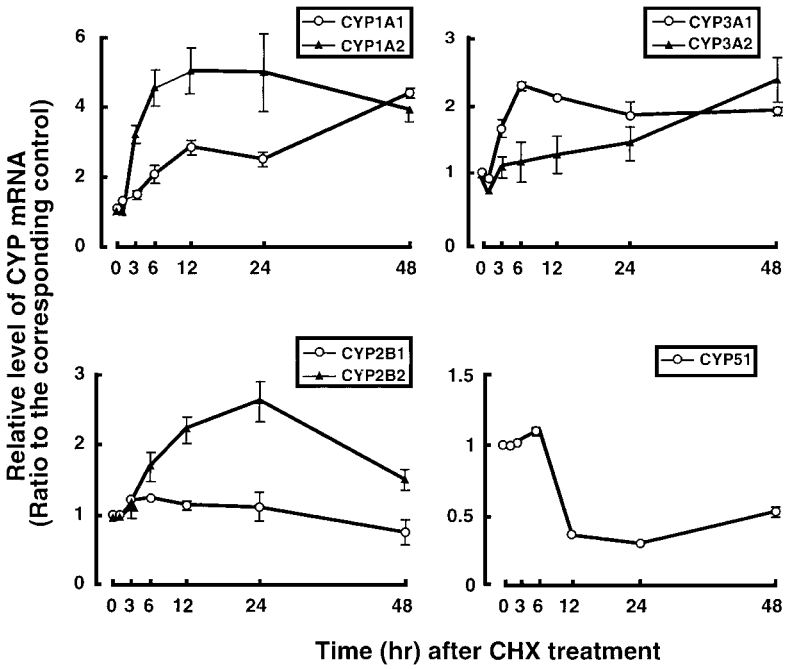

Fig. 6. **Time-dependent changes in the expression levels of several P450 genes after treatment of Kan-R2 cells with CHX.** Total RNAs were prepared from Kan-R2 cells as described in the legend to Fig. 5, and used the RT-PCR analysis. The expression levels of P450 genes were calculated on the basis of that of the *GAPDH* gene, and compared to the corresponding controls (CHX-untreated Kan-R2 cells). The values shown are the means for triplicate experiments, and bars represent the standard deviation of the means.

1) or 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) (data not shown). Thus, it is probable that the difference between 3-MeO-AAB and two other aromatic amines in the capacity to induce proliferous hepatic cells is due to the levels of the enzymes responsible for metabolic activation of the hepatocarcinogens. This is assumed from the facts that the metabolic activation of Glu-P-1 and Trp-P-1 (*28*) is mediated primarily by CYP1A2, which is not detected in primary cultured hepatocytes (*29*), but that of 3-MeO-AAB is mediated not only by CYP1A2 but also by other P450 isoforms (*28*).

The cultured cell lines, Kan-R1 and Kan-R2, showed similar characteristics in the expression pattern of P450 genes, although these cell lines differed with regard to cell size and colony-forming capacity on soft agar. The present findings suggest that the malignancy of the cells is not necessarily correlated with their ability to express the P450 genes.

The expression levels of the *CYP1A1* gene in Kan-R1 and Kan-R2 cells were higher than those of liver tissues, whereas those of the *CYP1A2, CYP2B1, CYP2B2, CYP3A1,* and *CYP3A2* genes were lower. These findings indicate that there are quantitative and/or qualitative difference(s) between the cultured cell lines and liver tissues in the cellular factors responsible for transcriptional regulation of the P450 genes.

It has been reported that the expression level of the *CYP1A1* gene was higher in primary cultured hepatocytes (*29*) and carcinogen-induced liver hyperplastic nodules (*30*, *31*) than in normal liver tissue, whereas that of the *CYP1A2* gene was the opposite. Thus, increased gene expression of CYP1A1 in cultured hepatic cells and liver hyperplastic nodules occurs through a reduction of a negative transcription factor(s) and/or ligand (xenobiotic)-independent activation of the aryl hydrocarbon receptor (AhR) responsible for xenobiotic-mediated CYP1A1 and CYP1A2 induction (*32*). Considering the decreased expression of the *CYP1A2* gene in Kan-R1 and Kan-R2 cells, as compared to in liver tissues, the increased expression of the *CYP1A1* gene in these cell lines seems to occur through a reduction of the negative transcriptional factor(s) for the gene rather than ligand-independent activation of AhR. The decreased expression of the *CYP1A2* gene in the cell lines might occur through an increase in the negative transcription factor(s) for the gene. These hypotheses were supported by the findings that the culture of Kan-R1 and Kan-R2 cells in the presence of CHX, an inhibitor of protein biosynthesis, resulted in increases in the gene expression levels of CYP1A subfamily enzymes, especially CYP1A2. Namely, negative transcription factors are thought to act as primary regulators for constitutive gene expression of the CYP1A subfamily enzymes, especially CYP1A2, in Kan-R1 and Kan-R2 cells. In addition, since the expression level of the *CYP1A1* gene synergistically increases with simultaneous treatment with CHX and CYP1A1 inducer in the rat liver (*33–36*), the negative transcription factor(s) for the *CYP1A1* gene should be considered as a regulatory factor in CYP1A inducer-mediated gene activation.

Furthermore, we demonstrated the expression of the *AhR* gene in both Kan-R1 and Kan-R2 cell lines. Our preliminary experiments, however, revealed that treatment of Kan-R1 and Kan-R2 with 3-methylcholanthrene, an inducer of both CYP1A1 and CYP1A2 in the rat liver (*8*), resulted in activation of the *CYP1A1* gene but not the *CYP1A2* gene, and that 3-MeO-AAB, a selective inducer of CYP1A2 in the rat liver (*27*), could not activate the *CYP1A2* gene in the cell lines (data not shown). These findings indicate that both Kan-R1 and Kan-R2 cells have the positive transcription factors including AhR for CYP1A inducer-mediated gene activation of CYP1A1, whereas they have lost the essential factor(s) for *CYP1A2* gene activation and/or over-express negative factor(s) for the gene.

Although the mechanism underlying the constitutive gene expression of CYP2B and CYP3A subfamily

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enzymes remains unclear, nuclear transcription factors such as constitutive androstane receptor (CAR), retinoid X receptor (RXR), and pregnane X receptor (PXR) have been reported to play important roles in xenobiotic-mediated gene activation; the RXR-CAR heterodimer for the *CYP2B* genes (*37*, *38*), and the RXR-PXR heterodimer for the *CYP3A* genes (*39*, *40*). Therefore, we examined whether the genes of transcription factors such as CAR, RXR, and PXR are expressed or not in Kan-R1 and Kan-R2 cells. No expression of the *CAR* gene and clear expression of the *RXR* gene were observed in either Kan-R1 or Kan-R2, although the genes of CYP2B1 and CYP2B2 were constitutively expressed in both cell lines, suggesting that CAR might not be an essential factor for constitutive gene expression of the CYP2Bs. On the other hand, Kan-R1 and Kan-R2 cells slightly expressed the *PXR* gene, and also slightly the genes of CYP3A1 and CYP3A2, suggesting that PXR might act, at least in part, as a positive transcription factor for the constitutive expression of the *CYP3A* genes. In addition, our preliminary experiments revealed that in neither Kan-R1 nor Kn-R2, gene expression of the CYP2B subfamily enzymes was enhanced by phenobarbital, an inducer of CYP2B1 and CYP2B2 in the rat liver (*41*) (data not shown), indicating that CAR is an essential transcription factor for the xenobiotic-mediated gene activation of CYP2Bs. Likewise, no enhanced gene expression of CYP3A subfamily enzymes with dexamethasone, an inducer of CYP3A1 and CYP3A2 in the rat liver (*41*), was observed in either Kan-R1 or Kan-R2 cells (data not shown), and the loss of responsiveness to the inducer might be due to only slight expression of the *PXR* gene in the cell lines. Furthermore, culture of Kan-R1 and Kan-R2 cells in the presence of CHX, an inhibitor of protein biosynthesis, resulted in definite increases in the expression levels of the *CYP2B2, CYP3A1*, and *CYP3A2* genes, but not in that of the *CYP2B1* gene, suggesting that negative transcription factors act as primary regulators for the constitutive expression of these P450 genes in the cell lines.

Considering all the findings in the present study, the constitutive expression of the P450 genes, *CYP1A1, CYP1A2, CYP2B2, CYP3A1, and CYP3A2*, in Kan-R1 and Kan-R2 cells seems to be controlled by the levels of negative transcription factors rather than those of positive factors, because their expression levels were increased by CHX, a non-selective inhibitor for the synthesis of proteins including negative and positive transcription factors. Namely, the decreased expression of the *CYP1A2, CYP2B2, CYP3A1,* and *CYP3A2* genes in Kan-R1 and Kan-R2 cell lines, as compared to in liver tissues, can be attributed to the increased levels of negative transcription factors. On the other hand, the decreased expression of the *CYP2B1* gene might occur through a different mechanism, because no CHX-mediated activation of the gene was observed. In addition, expression of the *CYP51* gene, which codes for lanosterol 14α-demethylase responsible for cholesterol biosynthesis, in both Kan-R1 and Kan-R2 cells was suppressed by CHX. Thus, differences among the CYP genes in the expression pattern after CHX treatment indicate that CHX-mediated increases in the expression levels of the *CYP1A1, CYP1A2, CYP2B2, CYP3A1,* and *CYP3A2* genes do not occur through stabilization of the mRNAs.

In conclusion, we succeeded in establishing the cultured rat hepatic cell lines, Kan-R1 and Kan-R2, that constitutively express several P450 genes, and further suggested that a negative transcription factor plays an important role in the constitutive gene expression of each P450 isoform, CYP1A1, CYP1A2, CYP2B2, CYP3A1, or CYP3A2. The newly established cell lines, Kan-R1 and Kan-R2, will be useful for investigation of the regulation mechanisms for the genes of CYP1A, CYP2B, and CYP3A subfamily enzymes.

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