Cell-density-dependent Expression of *Cyp*1a2 Gene in Monolayer-cultured Adult Mouse Hepatocytes

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Expression of *Cyp*1a1 and *Cyp*1a2 genes was investigated in adult C57BL/6NCrj mouse hepatocytes for up to 5 days after transfer to monolayer culture. CYP1A1 mRNA was substantially induced by treatment with 3-methylcholanthrene during the observation period, independently of the seeded cell density. However, expression of CYP1A2 mRNA was dependent on cell density and was higher in cells cultivated at lower density. With increasing culture period the expression was decreased, so that only negligible levels were evident by day 5, and reduced expression of constitutive and induced CYP-1A2 mRNA became apparent earlier in more densely seeded cells. This was not related to differences in numbers of inducer molecules per cell. While mouse hepatocytes incorporated tritium-labeled thymidine under the given culture conditions, induction of expression of the two *Cyp*1a genes did not show any direct relationship with DNA synthesizing activity. These observations suggest some role for *Cyp*1a2 during changes in physiological state.

Key words: Mouse hepatocyte — CYP1A1 — CYP1A2 — Cell density

Most chemical carcinogens require metabolic activation before exerting their biological activities and several kinds of cytochrome P-450 species have been demonstrated to catalyze this process for specific carcinogens.[1-6] P4501A2 mediates hydroxylation of aromatic amines, heterocyclic amines and azo compounds, and the products are further metabolized to the ultimate carcinogenic forms by additional modification, such as sulfation and acetylation.[7-9] P4501A2, which is constitutively expressed, is also inducible by treatment with aromatic hydrocarbons only in the liver *in vivo*.[6] However, with the usually employed method of hepatocyte primary culture, the content of P4501A2 protein decreases rapidly when the cells are transferred to monolayer culture and induction has not been successful except in limited cases.[10-13] Induction of CYP1A1 mRNA is, in contrast to CYP1A2, relatively easy in cultured cells, including monolayer hepatocytes, and several regulatory factors, such as a short-lived suppressor protein[14-17] and cAMP,[18] have been proposed. However, regulatory mechanisms during *Cyp*1a2 gene expression are still unclear and, since the P4501A2 protein is thought to be responsible for activation of human-related chemical carcinogens, it is of importance to elucidate the relationship of its expression to cellular physiological processes such as differentiation and replication.

The present paper deals with expression of *Cyp*1a2 and *Cyp*1a1 genes in monolayer-cultured mouse hepatocytes seeded at different cell densities. The results, while showing an apparent decrease in *Cyp*1a2 gene expression with increasing culture period also demonstrate that induction of the *Cyp*1a2 gene is inversely dependent on seeded cell density, that of the *Cyp*1a1 gene being independent. Furthermore, changes in the level of expression did not correlate with proliferation activity.

MATERIALS AND METHODS

Chemicals Materials for culturing hepatocytes were purchased from GIBCO Laboratories, Grand Island, NY, Collaborative Research Inc. Bedford, MA, and Kyokuto Seiyaku, Tokyo. Percoll and collagenase were products of Pharmacia, Uppsala, and Sigma Chemical Co., St. Louis, MO, respectively. *Cyp*1a1 and *Cyp*1a2 cDNAs cloned from mouse liver[19, 20] were generous gifts from Dr. Daniel W. Nebert, Univ. of Cincinnati, OH. Chicken β-actin cDNA was obtained from Oncor, Inc., Gaithersburg, MD. 3-Hydroxybenzo[*a*]pyrene was supplied by the NCI Chemical Repository, Bethesda, MD. Other routine chemicals were purchased from Seikagaku Kogyo, Dai-ichi Pure Chemicals, and Wako Pure Chemicals, Tokyo.

Preparation of primary cultures of hepatocytes The livers from female C57BL/6NCrj mice weighing 25–30 g (Charles River Japan, Inc.) were subjected to collagenase perfusion and then Percoll isodensity centrifugation for isolation of viable hepatocytes using the method previously described.[17, 21] The cells were dispersed in Waymouth MB752/1 medium containing bovine serum albumin (2 g/liter), insulin (0.5 mg/liter), transferrin

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Abbreviations used are: cDNA, complementary DNA; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

(0.5 mg/liter), selenium (0.5 μg/liter) and dexamethasone (0.4 μg/liter), and seeded in dishes precoated with 100 μg collagen Type I, at a density of 0.5 to 4.0×10^6 cells/10 ml/100 mm dish, unless otherwise indicated. The cultures were maintained at 37°C in a humidified CO_2 incubator. Medium was renewed every day, except in the experiments for measuring DNA synthesizing activity, but, since medium change in itself provoked *Cyp*1a1 gene expression.[21, 22] treatment with inducers was started 24 h after the last medium change. The inducers were dissolved in dimethyl sulfoxide, which itself had no influence on aryl hydrocarbon hydroxylase (a marker enzyme for *Cyp*1a1 gene) expression at the routinely employed maximal concentration of 0.1%.

Hybridization of hepatocyte RNA with P450 probes Total RNA was prepared from the hepatocytes according to the guanidinium thiocyanate/phenol method[23] and subjected to hybridization as described by Maniatis *et al.*[24] Northern transfer experiments were performed after size-fractionation of the denatured RNA (10-20 μg) on formaldehyde containing 1.3% agarose gels. Hybridization was carried out at 42°C overnight in a mixture containing 50% formamide, $1 \times$ Denhardt, $5 \times$ SSC, 50 mM sodium phosphate, pH 6.4, salmon testis DNA at 0.25 mg/ml, and a ^{32}P-labeled cDNA probe. Washing was performed four times for 5 min with $2 \times$ SSC and 0.1% SDS at room temperature, and then twice for 15 min with $0.1 \times$ SSC and 0.1% SDS at 60°C. Exposure to Kodak X-ray film was carried out at -70°C with an intensifying screen (Du Pont).

Incorporation of thymidine into acid-insoluble fraction DNA synthesizing activity of hepatocytes was determined essentially as described earlier.[25] The last medium change before determination of DNA synthesizing activity was carried out 24 h after the seeding. [Methyl-^{3}H]-thymidine (Amersham Japan, Tokyo) was added to the culture medium at 1 μCi/ml. After 1 h cultivation, dishes were washed several times with phosphate-buffered saline and then with cold 10% trichloroacetic acid. The cells were then dissolved in 1 ml of 0.5 N NaOH. The resultant solution was transferred to a centrifuge tube on ice and mixed with 0.1 ml of cold 100% trichloroacetic acid. The precipitate was suspended in 10% trichloroacetic acid after centrifugation of the mixture, followed by boiling for 15 min. Tritium-radioactivity in the supernatant fraction was determined after neutralization with the aid of a Triton-toluene liquid scintillator.

RESULTS

Induction of *Cyp*1a1 and *Cyp*1a2 gene expression at different cell densities Total RNAs were prepared daily from mouse hepatocytes, which were seeded at 5×10^5 cells on either 60 mm (21 cm^2) or 100 mm (55 cm^2) dishes and
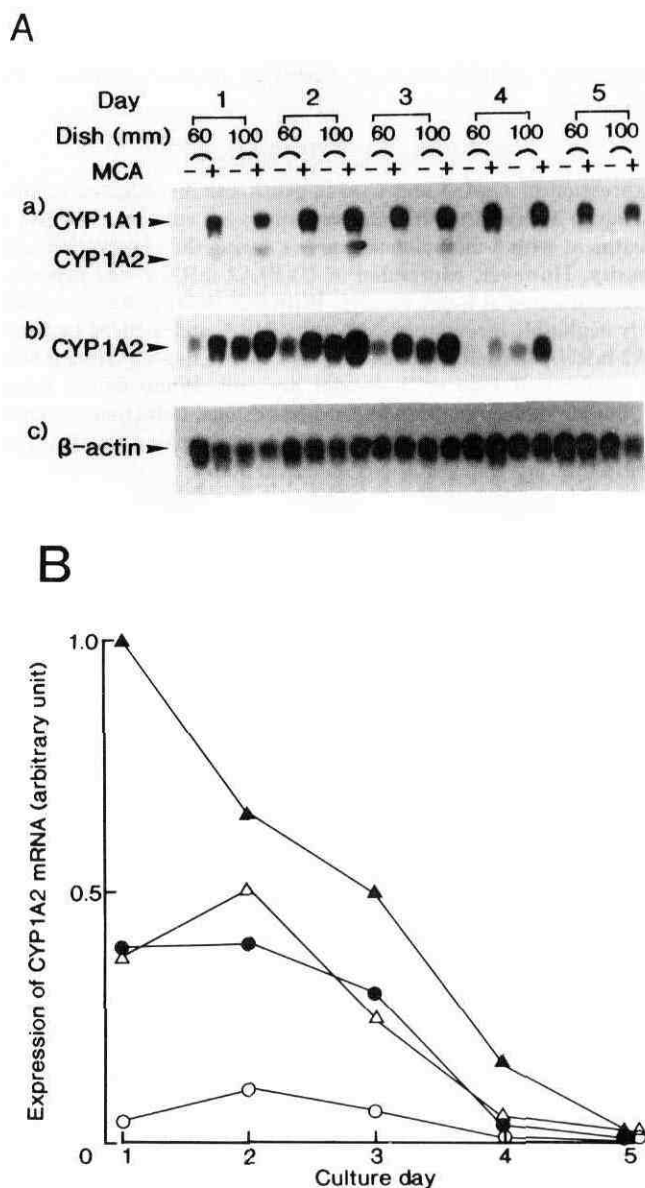

Fig. 1. Daily change in CYP1A1 and CYP1A2 mRNA expression after treatment of monolayer-cultured hepatocytes at different cell densities with 3-methylcholanthrene. Mouse hepatocytes (5×10^5) were seeded on collagen-coated 60 or 100 mm dishes, and treated with 1.625 μM 3-methylcholanthrene (MCA) at the indicated culture time (e.g. day 3 means that the treatment started 72 h after the seeding). A) Northern-blot hybridization of total RNA prepared 10 h after the start of the treatment was performed using cDNA probes for mouse *Cyp*1a1 (a) and *Cyp*1a2 (b), and chicken β-actin (c). The same filter was used for all hybridizations. B) Amounts of expressed CYP1A2 mRNA were determined by densitometry. The amounts were corrected on the basis of those of actin mRNA. Each point represents the mean from duplicate experiments. ($\bigcirc$): 60 mm dish control, ($\bullet$): 3-methylcholanthrene-treated. ($\triangle$): 100 mm dish control, ($\blacktriangle$): 3-methylcholanthrene-treated.

Cell density (×10⁴cells/cm²) ... 0.91 1.82 3.64 7.27
MCA(µM) 1.625 13 1.625 13 1.625 13 1.625 13
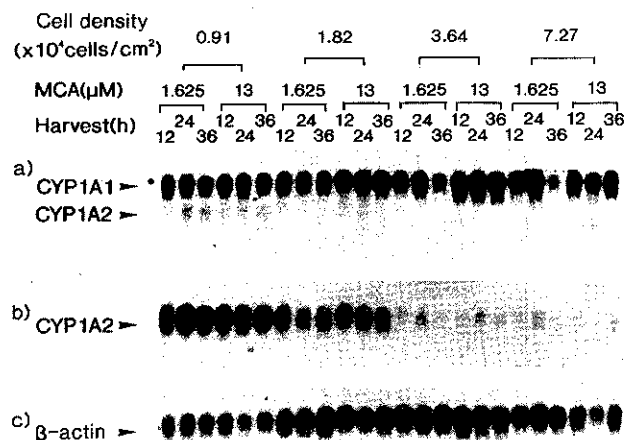Harvest(h) ...

a) CYP1A1 CYP1A2
b) CYP1A2
c) β-actin

Fig. 2. Induction of *Cyp*1a genes by 3-methylcholanthrene in hepatocytes at different cell densities. Mouse hepatocytes at day 3 were treated with either 1.625 or 13 μM 3-methylcholanthrene (MCA). The cells were seeded at 0.91, 1.82, 3.64 and 7.27×10^4 cells/cm². Total RNA was prepared at the indicated times after the start of the treatment. Probes were the same as described in the legend to Fig. 1.

Inducer no MCA TCDD IS
Cell density (×10⁴cells/cm²) 0.91 7.27 0.91 7.27 0.91 7.27 0.91 7.27
Harvest(h) ...

a) CYP1A1 CYP1A2
b) CYP1A2
c) β-actin

Fig. 3. Expression of *Cyp*1a genes after treatment with 3-methylcholanthrene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or isosafrole. Hepatocytes seeded at either 0.91 or 7.27×10^4 cells/cm² were treated at day 3 with either 1.625 μM 3-methylcholanthrene (MCA), 3.1 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or 3 mM isosafrole (IS). Total RNA was prepared at the indicated harvest times after addition of the chemicals. Northern-blot hybridization was carried out with the same probes as employed for Fig. 1.

treated for 10 h with 1.625 μM 3-methylcholanthrene. Northern-blot hybridization of the RNAs revealed considerable amounts of CYP1A1 mRNA after this treatment during the observation period of up to 5 days, independent of culture dish size, and with a peak around days 2–4 (Fig. 1). Constitutively expressed CYP1A2 mRNA decreased with increasing culture period, but the reduction in the 60 mm dish cases was more rapid than in the 100 mm dishes. Induction of CYP1A2 mRNA by 3-methylcholanthrene was substantial within the first 2 or 3 days of cultivation, and the induced amounts were smaller in cells seeded at the higher density, resulting in expression almost equivalent to the constitutive levels of the cells at the lower density. The induction drastically decreased to negligible levels by day 5, but this process proceeded at a slower rate in cells at the low density. Levels of β-actin mRNA were not influenced by cell density or by treatment with 3-methylcholanthrene.

Expression of *Cyp*1a genes at different concentrations of 3-methylcholanthrene Figure 2 illustrates the induction of the two *Cyp*1a genes by various concentrations of 3-methylcholanthrene at day 3 of cultuvation. With 13 μM 3-methylcholanthrene, induction of CYP1A1 mRNA in hepatocytes was prominent, being similar in magnitude at different cell densities. With 1.625 μM no difference was observed in induction of this mRNA species at 12 or 24 h after the inducer treatment, but at the 36 h time point a decrease was observed with increasing cell density. In contrast, expression of CYP1A2 mRNA
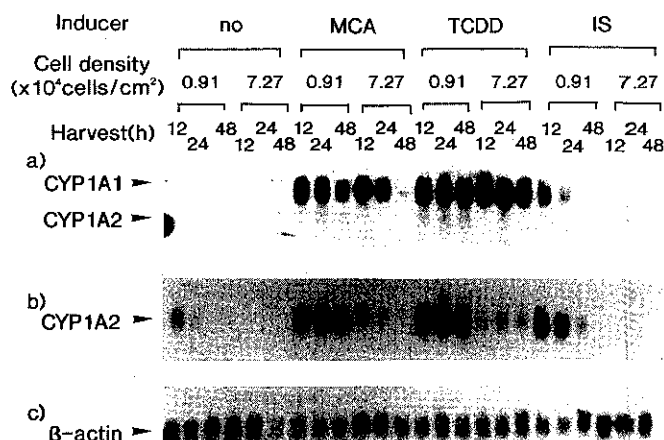
was highly dependent on cell density and was greatest at 0.91×10^4 cells/cm², followed by 1.82×10^4 cells/cm². No marked induction of CYP1A2 mRNA was found at higher cell densities with both concentrations of 3-methylcholanthrene, indicating that, even with essentially equal numbers of inducer molecules per hepatocyte, the inductive response of the *Cyp*1a2 gene was dependent on cell density. Expression of β-actin mRNA again proved to be almost constant, independent of cell density.

Expression of *Cyp*1a gene by TCDD or isosafrole Figure 3 compares expression of CYP1A1 and CYP1A2 mRNAs after treatment with various typical inducers. Both TCDD (3.1 pM) and isosafrole (3 mM) induced CYP1A2 mRNA in cells at low density to nearly the same extent at 3-methylcholanthrene (1.625 μM), although an earlier decrease with time was found after isosafrole treatment. In contrast, CYP1A2 mRNA expression in cells at high density was very weak. Induction of CYP1A1 mRNA was at higher levels after TCDD treatment than after 3-methylcholanthrene or isosafrole. While cell density did not affect the maximal induction of this gene after 3-methylcholanthrene or TCDD treatment, the decrease over time was faster after 3-methylcholanthrene in cells at high density. Isosafrole did not induce CYP1A1 mRNA in cells at high density, but a clear hybridizable band was observed in cells at low density.
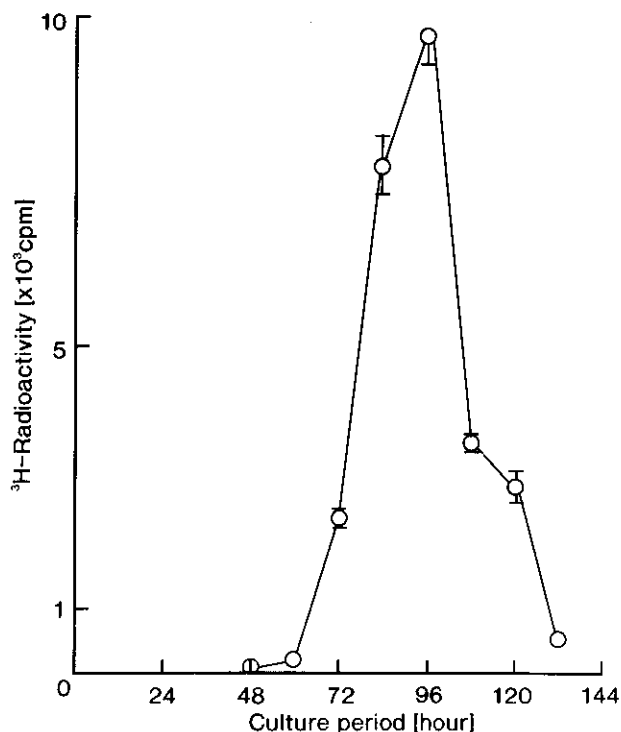
Fig. 4. DNA synthesis in mouse hepatocytes in primary culture. Mouse hepatocytes were subjected to medium change at 24 h after seeding at 2.38×10^4 cells/cm². Tritium-labeled thymidine was added at 1 μCi/ml and incorporation of radioactivity into the acid-insoluble fraction was determined 1 h later as indicated in the figure. Each point represents the mean ± SD from triplicate experiments.
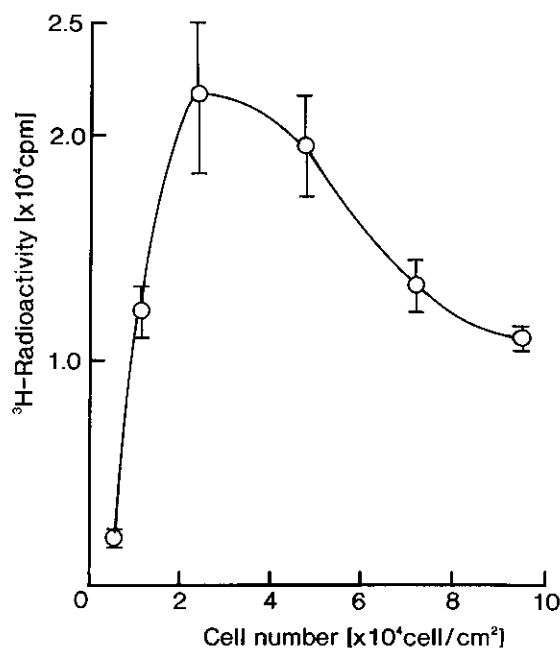

Fig. 5. Dependency of DNA synthesis of mouse hepatocytes on cell density. Hepatocytes seeded at the indicated densities were assayed for their DNA synthesizing activity at 95–96 h after seeding by measuring incorporation of tritium-labeled thymidine into the acid-insoluble fraction. Each point represents the mean ± SD from triplicate experiments.

Cell-density-dependent DNA synthesis DNA synthesizing activity was determined by assaying radioactivity incorporated into the acid-insoluble fraction after a 1 h treatment of hepatocytes with ³H-thymidine. Although rat hepatocytes require the presence of either epidermal or hepatocyte growth factor for initiating DNA synthesis,[26] mouse cells incorporated thymidine at quite high levels without such factors, namely under the same culture conditions as used for investigating Cyp1a gene expression. As shown in Fig. 4, DNA synthesis started at 60 h after seeding, peaking at 96 h, and thereafter drastically decreasing with a shoulder at 120 h. Activity of DNA synthesis was dependent on cell density with the highest level observed at a density of around 2×10^4 cells/cm² (Fig. 5).

DISCUSSION

The present investigations revealed that expression of the Cyp1a2 gene is highly dependent on seeded cell den-

sity in monolayer-cultured mouse hepatocytes, whereas that of Cyp1a1 gene is relatively density-independent. DNA synthesizing activity was also affected by cell density, but no correlation with expression of the Cyp1a2 gene could be demonstrated.

Although both Cyp1a1 and Cyp1a2 genes in vivo are inducible by the same kinds of aromatic hydrocarbons, it is considered that the mechanisms of regulation of the induction may be different in the two cases. One supporting line of evidence from the analysis of the Cyp1a2 gene nucleotide sequence is that no xenobiotic responsive elements, presumed to act as a binding region for the complex of the so-called Ah receptor with the inducer in Cyp1a1 gene,[27, 28] are apparent in the proximal 5′-flanking region.[29] Regulation of Cyp1a1 gene expression has been studied extensively and several regulatory factors have been suggested, such as a short-lived suppressor protein[14–17] and cyclic AMP.[18] Knowledge of Cyp1a2 gene expression is more limited, the main reason being that a convenient cell culture system for its investigation has hitherto not been available. The liver is the only organ which constitutively expresses Cyp1a2 gene and is highly responsive to its induction, and only slight expression of the mRNA can be detected when selected

extrahepatic tissues are treated with very high concentrations of the potent inducer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.[30] Hepatocytes in primary culture have nevertheless not been considered as a suitable tool for investigation of *Cyp*1a2 gene expression, because of the rapid decline of its constitutive expression and its responsiveness to inducers.[31–33] In 1988, however, two reports[10, 11] appeared on induction of CYP1A2 mRNA by 3-methylcholanthrene using rat hepatocytes in monolayer culture, describing its expression to be regulated primarily at the post-transcriptional level by mRNA accumulation. Subsequently, Sinclair *et al.* observed induction and accumulation of P4501A2 protein after administration of 3,4,5,3′,4′,5′-hexachlorobiphenyl to mouse hepatocytes cultivated on Matrigel™ in the presence of 5-aminolevulinic acid.[34] They considered that the accumulation could be due to stabilization of the induced P4501A2 protein by binding with the inducer. However, no attention has been paid to the influence of cell density on the expression of any P450 species. Cells in a confluent state have usually been employed, because biochemical analysis requires a certain volume of cells and tightly packed cells are thought to demonstrate diminished proliferation activity, suggesting them to be an appropriate tool for investigation of differentiated functions.

After transfer of hepatocytes to monolayer culture the physiological conditions are considerably changed. One reason why constitutive and inductive expression of the *Cyp*1a2 gene is rapidly lost is considered to be the acquisition of proliferation potential of the cells. In fact, maintenance of rat hepatocytes at low density remains the basic method for studying growth. Since cells in tightly packed confluent monolayers exhibit liver-specific differentiated functions, a cell-density-dependent reciprocal relationship is generally accepted between differentiation and DNA synthsis.[26, 35] Considering the substrate specificities of *Cyp*1a gene products, they might be expected to play their roles in the differentiated state, although slight expression of both *Cyp*1a genes during neonatal developmental stages in the liver or in mouse embryos and retinoic acid-exposed F9 mouse embryonal carcinoma cells was reported by Kimura *et al.*[36] They suggested that the *Cyp*1a1 gene may have an important developmental function during differentiation, since activation of this gene can occur in the absence of a foreign chemical stimulus. However, the present results indicate that close cell-to-cell contact may not be required for induction of either of the *Cyp*1a genes, and that *Cyp*1a1 gene expression may not be correlated with either differentiated state or cell proliferation. In the present experiments prominent induction of CYP1A2 mRNA was observed in cells at lower than 1.8×10^4 cells/cm^2, which is an exceptionally low density as compared with those used in previous studies. Since mouse hepatocytes, once anchored to dish surfaces, spread more than rat cells, the cell density in routine use is around $0.8–1.5 \times 10^6$ cells/60 mm dish ($3.8–7.1 \times 10^4$ cells/cm^2), approximately half that of rat cells. The quite low levels of CYP1A2 mRNA induction after 2 days of cultivation, observed in other laboratories,[10, 11, 34] might be explained by our findings.

Kimura *et al.*[36] earlier observed alteration of the expression of *Cyp*1a1 and *Cyp*1a2 genes after partial hepatectomy in the mouse, namely decrease in constitutive CYP1A2 mRNA and increase in CYP1A1 mRNA, indicating that switching on of the *Cyp*1a2 gene reflects a differentiated function not observed during liver regeneration or cell division, while the opposite is the case for *Cyp*1a1 gene. Since the present experiments concerned inducer effects, the results cannot be directly compared with those of Kimura *et al.*[36] However, we could not find any suggestion of a relationship between DNA synthesis and expression of either of the two *Cyp*1a genes in response to 3-methylcholanthrene treatment. Moreover, though activation of chemical carcinogens may be considered as a differentiated function, our results did not suggest that these genes are involved in cellular differentiation. Elucidation of their significance awaits a better understanding of their linkage with physiologically important genes, the expression of which is coordinated with *Cyp*1a gene regulation during cultivation.

ACKNOWLEDGMENTS

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