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ALTERED REGULATION OF *Cyp*1a-1 GENE EXPRESSION DURING CULTIVATION OF MOUSE HEPATOCYTES IN PRIMARY CULTURE

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Abstract—Alterations in *Cyp*1a-1 gene expression in adult C57BL/6 mouse hepatocytes were followed after transferring them to primary culture during the initial 5 days. Changing the medium to a fresh one was associated with considerable amounts of *Cyp*1a-1 gene mRNA with a peak at around 6 hr after the medium change, followed by a decrease to negligible levels 24 hr later. Treatment of hepatocytes with cycloheximide increased the medium change-associated mRNA expression, the levels being equivalent to those observed after treatment with 3.2–25.6 nM 3-methylcholanthrene plus cycloheximide. With increasing length of culture period, cycloheximide-aided enhancement of the medium change-associated mRNA transcription increased. Although the chemical alone did not induce *Cyp*1a-1 gene transcripts in hepatocytes at day 1 or 2 of cultivation, for which medium had been changed 24 hr previously, prominent induction of transcripts was evident at later periods, the levels being elevated in accordance with length of time in culture. To examine whether or not the mRNA transcribed under these culture conditions was translatable, the cells were treated with actinomycin D after washing out the cycloheximide, in order to inhibit degradation of the generated mRNA (Nemoto N and Sakurai J, *Carcinogenesis* **12**: 2115–2121, 1991). After these procedures significant elevation of aryl hydrocarbon hydroxylase activity was observed in hepatocytes, the rise being well correlated with elevated levels of mRNA transcripts. The observations suggest that the *Cyp*1a-1 gene might be expressed at low levels during the initial phase of cultivation of mouse hepatocytes in primary culture. Whether this expression might be essential for mouse hepatocytes to adapt to culture conditions is unclear. The findings do suggest, however, that superinducibility of the gene expression after cycloheximide treatment might be a result of a regulatory mechanism operating after adaptation to culture.

Microsomal P450-dependent monooxygenases play important roles in the metabolism of exogenous as well as endogenous substances [1, 2], and purification and isolation of enzyme proteins, DNAs and cDNAs for several P450 species have allowed definition of their substrate specificities [3–9], some being found to activate environmental carcinogens. The CYP1A1 protein species is responsible for the activation of carcinogenic polycyclic aromatic hydrocarbons and its representative enzyme is aryl hydrocarbon hydroxylase (AHH†). Its activity can be induced by treatment with several kinds of aromatic hydrocarbon or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [10]. Although the precise induction mechanism remains to be elucidated, it has been proposed that the induction is initiated by the interaction of inducer with the cytosolic *Ah* receptor [11–13]. However, recent work utilizing the reverse transcriptase-polymerase chain reaction to amplify transcripts suggested constitutive expression of the corresponding mRNA in several organs of humans or non-treated rats [14]. Furthermore, the *Cyp*1a-1 gene was found to be expressed after partial hepatectomy and near delivery in the mouse [15, 16]. These observations suggest that the *Cyp*1a-1 gene may play some physiological role as one of the housekeeping genes. Several regulation mechanisms have been proposed for this gene, involving, for example, a short-lived repressor protein [17–19], cyclic AMP-dependent expression [20] or synthesis of poly(ADP-ribose) [21]. However, these regulation mechanisms have only been established in cultured cells and it remains uncertain whether or not they really work *in vivo*.

This paper deals with the expression of the *Cyp*1a-1 gene in the absence of any known so-called AHH inducer after the transferring of mouse hepatocytes to primary culture. While levels of mRNA expression were hardly detectable, cycloheximide treatment was associated with marked elevation especially with increasing culture period, suggesting that expression of this gene is very low level during cultivation under ordinary culture conditions and that superinduction of the gene by cycloheximide treatment might be gradually manifested as a result of adaptation to culture conditions.

MATERIALS AND METHODS

Chemicals. Materials for culturing hepatocytes were purchased from Gibco (Grand Island, NY, U.S.A.). Collaborative Research Inc. (Bedford, MA, U.S.A.) and Kyokuto Seiyaku (Tokyo, Japan). Percoll was a product of Pharmacia (Uppsala,

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† Abbreviations: AHH, aryl hydrocarbon hydroxylase; SDS, sodium laurylsulfate; SSC, standard saline citrate; Waymouth, Waymouth MB 752/1.

Sweden). Collagenase, cycloheximide and actinomycin D were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). *Cyp*1a-1 and *Cyp*1a-2 cDNAs cloned from mouse liver [22, 23] were generous gifts from Dr Daniel W. Nebert, University of Cincinnati, OH, U.S.A. 3-Hydroxybenzo-(*a*)pyrene was supplied by the NCI Chemical Repository (Bethesda, MD, U.S.A.). Rat albumin cDNA was obtained from the Japanese Cancer Research Resources Bank. ^{32}P-Labeled cytidine triphosphate and ^{3}H-labeled thymidine were purchased from ICN Biomedical Inc. (Costa Mesa, CA, U.S.A.). Other routine chemicals were purchased from Sei-Kakagu Kogyo, Dai-ichi Chemical and Wako Pure Chemicals (Tokyo, Japan).

Preparation of primary cultures of hepatocytes. The livers of female C57BL/6NCrj (Charles River, Japan) weighing 25–30 g were subjected to collagenase perfusion and then Percoll isodensity centrifugation for isolation of viable hepatocytes using the method described previously [24]. The cells were dispersed in Waymouth medium containing bovine serum albumin (2 g/L), insulin (5 mg/L), transferrin (5 mg/L), selenium (5 μg/L) and dexamethasone (4 μg/L) at a density of 0.5–1.0 × 10^6 cells/4 mL/60 mm dish, and allowed to seed in dishes coated with collagen type I. The cultures were maintained at 37° in a CO_2-humidified incubator. Medium was renewed every day unless otherwise specified. 3-Methylcholanthrene, an inducer, was dissolved in dimethyl sulfoxide, which itself had no influence on AHH activity at the routinely employed concentration of less than 0.1%.

Assay of AHH. The activity of AHH in hepatocytes was measured as described previously [24]. One milliliter of reaction mixture contained hepatocytes from one dish, 1.08 μmol NADPH, 3 μmol $MgCl_2$, 100 nmol benzo(*a*)pyrene and 50 μmol Tris–HCl, pH 7.5. The reaction was stopped after 30 min by addition of cold acetone and the metabolites extracted with *n*-hexane, followed by extraction with 1 N NaOH. Emission at 522 nm was measured with excitation at 396 nm using 3-hydroxybenzo(*a*)pyrene as a standard. Enzyme activity was expressed as picomoles 3-hydroxybenzo(*a*)pyrene produced per 10^6 cells per min. Student's *t*-test was employed for statistical evaluation of the data.

Hybridization of hepatocyte RNA with P450 probes. Total RNA was prepared from the hepatocytes according to the guanidinium thiocyanate/phenol method [25] and applied for hybridization as described by Maniatis *et al.* [26]. 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° overnight in a mixture containing 50% formamide, 1 × Denhardt, 5 × SSC, 50 mM sodium phosphate, pH 6.4, salmon testis DNA at 0.25 mg/mL, and ^{32}P-labeled cDNA probes for either mouse *Cyp*1a-1, *Cyp*1a-2 or rat albumin. Washing was performed four times for 5 min with 2 × SSC and 0.1% SDS at room temperature, and then twice for 15 min with 0.1 × SSC and 0.1% SDS at 60°. Exposure of Kodak X-ray film was carried out at −70° with an intensifying screen (Du Pont).

RESULTS

Induction of Cyp1a-1 *gene expression without an AHH inducer*

Figure 1 shows the results of northern-blot hybridization of total RNAs after medium change at day 3. Transcripts hybridizing to the band corresponding to *Cyp*1a-1 mRNA (2.9 kb) were clearly observed when the fresh medium employed was either Waymouth or D/F (1:1 mixture of Dulbecco's minimum essential medium and Ham F-12 medium). However, no transcripts were detected when the medium employed for the exchange was 1 day-cultured medium and the cells had been cultivated for 24 hr in either Waymouth or D/F medium, although 1 day-cultured Waymouth medium slightly stimulated transcript production in cells which had been cultivated in D/F medium. The medium change-associated expression of *Cyp*1a-1 gene was maximal around 3–6 hr after the procedure, followed by a decrease to an undetectable level 24 hr later. Constitutively expressed *Cyp*1a-2 mRNA (2.1 kb) was increased in linkage with the change in *Cyp*1a-1 gene expression.

Superinduction of Cyp1a-1 *gene expression by cycloheximide during cultivation*

Superinduction of transcripts by transient inhibition of protein synthesis has been reported as one of the regulatory mechanisms of *Cyp*1a-1 gene expression caused by so-called AHH inducers in several cultured cells [17–19]. Medium change-associated *Cyp*1a-1 gene transcription was also enhanced by treatment with cycloheximide (Fig. 2). With increasing length of culture period, medium change-associated *Cyp*1a-1 transcripts were expressed, and cycloheximide treatment further increased the levels of transcripts. Furthermore, although amounts of the transcripts were negligible 24 hr after the medium change, addition of cycloheximide at this time-point resulted in marked production of mRNA, when the culture period was 4 or 5 days (Fig. 3). However, when cycloheximide was added 24 or 48 hr after the start of cultivation no appreciable expression of the *Cyp*1a-1 gene was observed.

Comparison of medium change-associated Cyp1a-1 *gene expression with that induced by 3-methylcholanthrene treatment*

3-Methylcholanthrene induced *Cyp*1a-1 gene transcription with a peak in the amounts of transcripts occurring at 9 hr or later depending on the concentration employed (Fig. 4). With lower concentrations, amounts of the transcripts reached a peak at 9 hr and then decreased, while the peak times were delayed after treatment with high concentrations of the inducer. Medium change-associated and cycloheximide-aided expression of mRNA at day 3, when its levels were maximal, corresponded approximately with 3-methylcholanthrene treatment at between 3.2 and 25.6 nM.

AHH activity after treatment with cycloheximide and actinomycin D

In the case of inhibition of protein synthesis by

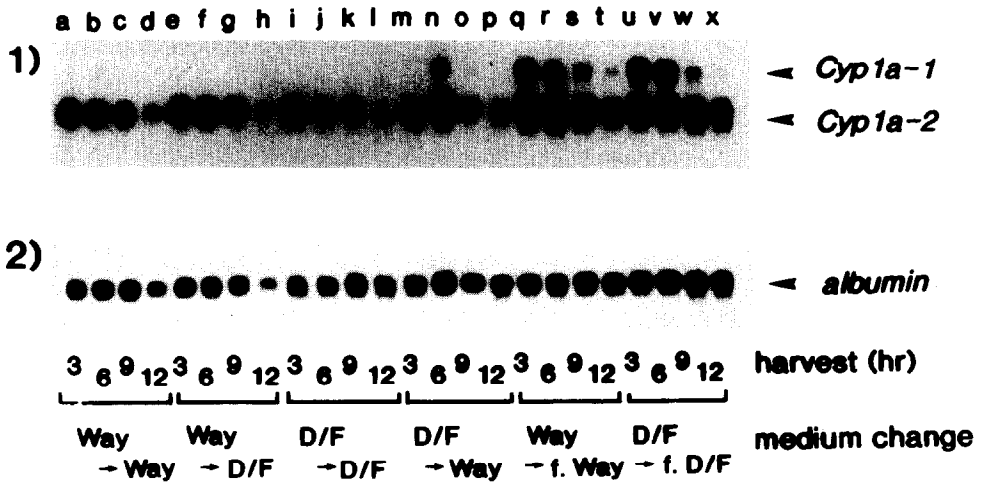

Fig. 1. Expression of *Cyp*1a-1 transcripts after medium change. Culture medium was changed to the indicated medium at day 3 after seeding. Total RNAs were prepared at the indicated harvest intervals after the medium change procedure and northern blot hybridization was performed using cDNA probes for mouse *Cyp*1a-1 and *Cyp*1a-2 (1), and rat albumin (2). The same filter was used for all hybridizations. Way, Waymouth MB 752/1; D/F, 1:1 mixture of Dulbecco's minimum essential medium and Ham F-12; f., fresh medium. Lanes a–p: medium employed for the exchange was a conditioned one, in which hepatocytes had been cultivated for 24 hr. Lanes q–x: fresh medium was used for the exchange.

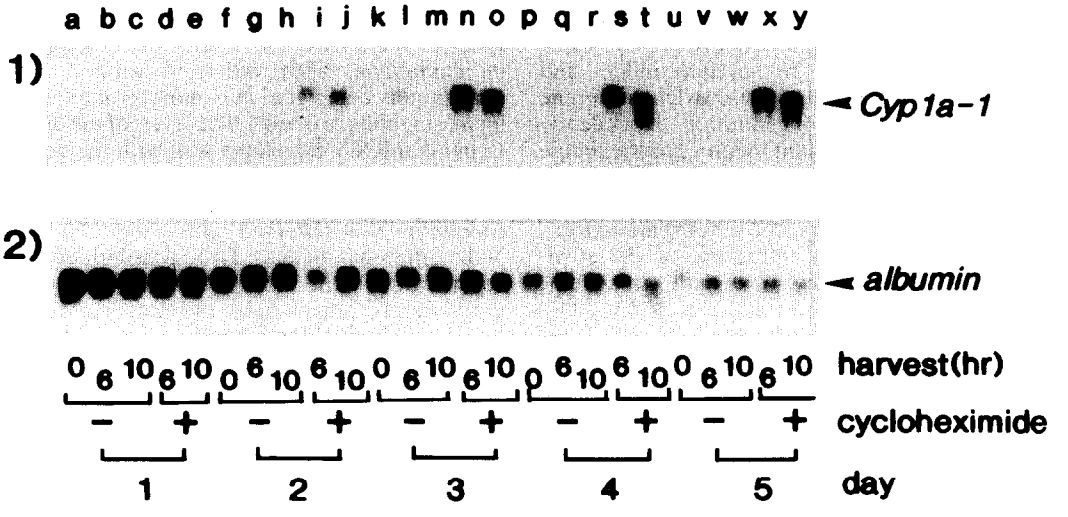

Fig. 2. Effect of cycloheximide on time-course of *Cyp*1a-1 gene expression after daily medium change. Medium was changed every day and cycloheximide at 1 μg/mL was concomitantly added to the medium on the harvest day. Total RNAs were extracted at each medium change, or 6 or 10 hr thereafter. For example, sample RNA for lane n was prepared 6 hr after medium change and cycloheximide addition at day 3. Probes were (1) *Cyp*1a-1 and (2) albumin.

cycloheximide, no appreciable increase in AHH activity was observed during the presence of this compound. However, a small but significant increase in enzyme activity was found within a few hours after removal of cycloheximide, the level being far lower than after treatment with so-called AHH inducer [27]. Therefore, the elevated levels of mRNA observed with cycloheximide did not correlate directly with enzyme activity. As a means of post transcriptional regulation of *Cyp*1a-1 gene

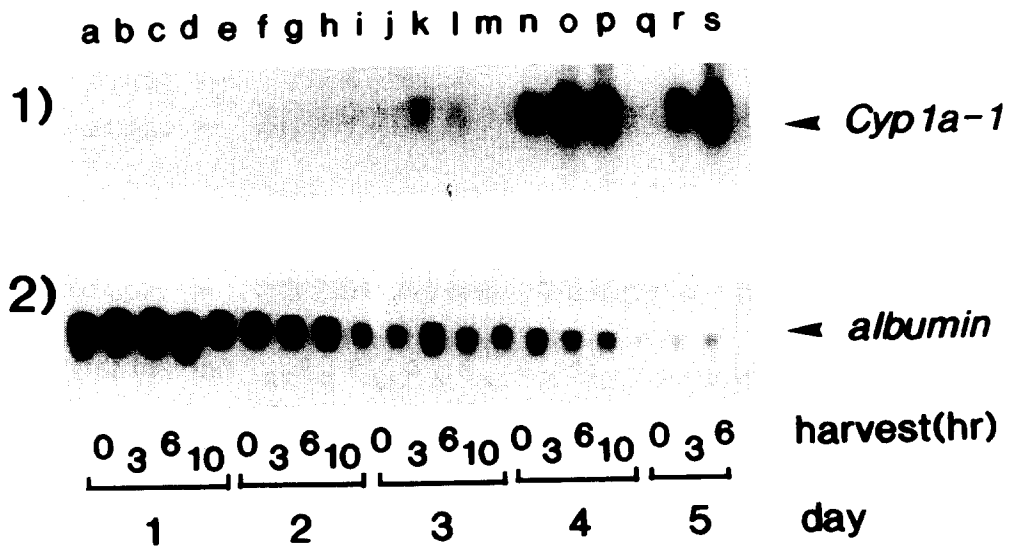

Fig. 3. Enhanced expression of *Cyp*1a-1 gene after treatment with cycloheximide. Medium was changed every day. Cycloheximide at 1 μg/mL was added to the medium, which had been changed 24 hr previously. Total RNAs were prepared at the indicated harvest intervals after start of exposure to cycloheximide. For example, RNA for lane n was prepared 3 hr after addition of cycloheximide without medium change on day 4. Probes employed were the same as for Fig. 2.

expression, we reported that inhibition of further RNA synthesis resulted in stabilization of the mRNA and thus superinduction of AHH activity [27]. Figure 5 demonstrates alteration in 3-methylcholanthrene-induced AHH activity by cycloheximide and actinomycin D treatments. 3-Methylcholanthrene induced AHH activity concentration dependently and concomitant treatment with cycloheximide decreased the enzyme activity during the early phase of cultivation. However, thereafter cycloheximide treatment significantly enhanced the enzyme activity, especially at lower concentrations of 3-methylcholanthrene. Without cycloheximide treatment, peak enzyme activities were attained at 13×8^{-1}–13×8^{-2} μM 3-methylcholanthrene, whereas this shifted to lower concentrations with increasing culture period, especially after incubation between 96 and 120 hr. The highest level of induced AHH activity either in the presence or absence of cycloheximide was found after incubation for between 48 and 72 hr. With increasing culture period prominent induction of AHH activity was also observed in hepatocytes not treated with 3-methylcholanthrene but after medium change (Table 1). Similarly to the *Cyp*1a-1 mRNA expression illustrated in Fig. 4, levels of AHH activity due to medium change corresponded approximately with those after 3-methylcholanthrene treatment at 3.2–25.6 nM. Significant elevation of AHH activity was observed with this technique in cells 96 or 120 hr after seeding, even though medium had been changed 24 or 48 hr previously (Tables 1 and 2).

DISCUSSION

The present examination of *Cyp*1a-1 gene expression in mouse hepatocytes during 5 days of a primary culture revealed that medium change provokes transient transcription of this gene and cycloheximide treatment results in enhancement of its expression, both with and without medium change, this being gradually more pronounced with increasing culture period. The levels of the expressed *Cyp*1a-1 mRNA correlated well with the activity of the correspond enzyme, AHH.

Medium change-associated *Cyp*1a-1 gene expression was observed with either the same or with different media. Two possibilities require consideration: the first is that specific medium constituent(s) might have the potential to induce AHH activity and the second is that the expression is a normal cellular response to replenishment of partly exhausted nutrients. Application of conditioned media provoked only a little or negligible amount of *Cyp*1a-1 gene transcripts, which could support either of these possibilities. What is certain is that conditioning of the medium greatly reduced whatever factor(s) were responsible for the *Cyp*1a-1 gene expression.

Some kinds of amino acid present in the medium could become AHH inducers after oxidation by UV illumination in the presence of riboflavin [28, 29]. However, under the present experimental conditions, whether production of such oxidized compounds actually occurs remains unknown and the question is difficult to resolve, because mouse hepatocytes require medium change for effective maintainance. The possibility cannot be disregarded that a low level of transcription after treatment with cycloheximide might be initiated by medium change carried out earlier.

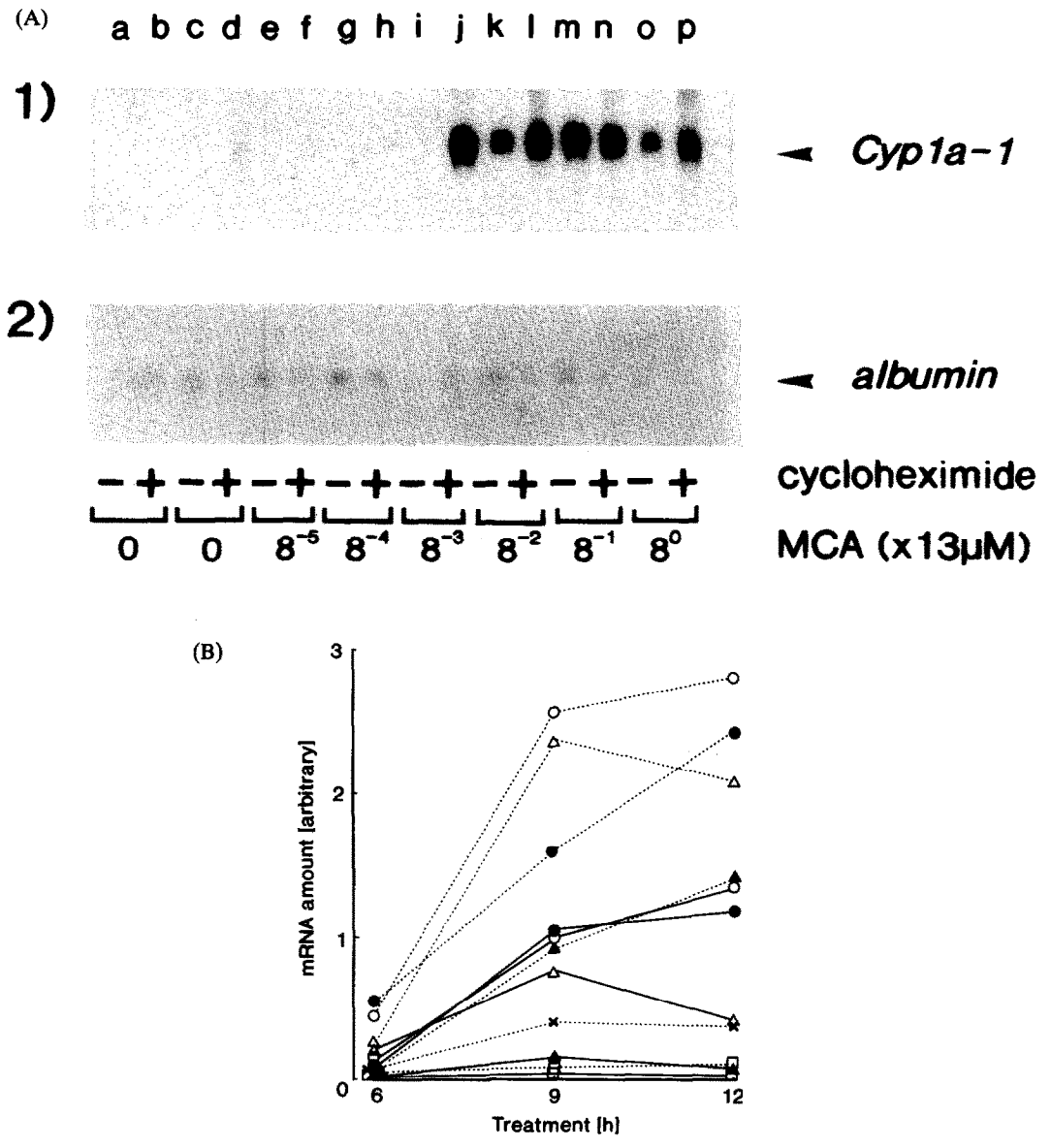

Fig. 4. Expression of *Cyp*1a-1 gene after treatment with various doses of 3-methylcholanthrene. Hepatocytes at day 3 were treated with the indicated amounts of 3-methylcholanthrene and/or 1 μg/mL cycloheximide without medium change. Total RNA was extracted at 6, 9 or 12 hr after addition of the chemical. (A) Northern-blot hybridization of total RNAs of hepatocytes, which were treated with 3-methylcholanthrene and/or cycloheximide for 9 hr. (B) Amounts of *Cyp*1a-1 gene transcripts plotted from mean densities of duplicate dot-blot hybridization experiments with a standard for 13 μM 3-methylcholanthrene-treated samples at the 9 hr level. Concentration of 3-methylcholanthrene: 13 μM × 1 (○), × 8^{-1} (●), × 8^{-2} (△), × 8^{-3} (▲), × 8^{-4} (□). Cycloheximide treatment: no (solid line), yes (dotted line). Medium change + cycloheximide (× · · · · ×).

In the present experiments, cycloheximide induction of *Cyp*1a-1 gene transcripts and elevation of AHH activities in hepatocytes, with medium change at either 0 or 24 hr previously, progressively increased with cultivation period. Cycloheximide itself has not been reported to have any AHH induction potency and this would be difficult to confirm *in vivo*, because of its inhibitory action on protein synthesis. However, since no enhancement of *Cyp*1a-1 gene transcription was observed during the early phase of cultivation and 3-methylcholanthrene induced transcripts even during this period, cycloheximide presumably does not initiate gene transcription in the same way. The results also indicate that mouse hepatocytes could express *Cyp*1a-1 gene at a low level during cultivation which might become detectable only after treatment with cycloheximide. We consider it possible that the gene coding for the putative short-lived suppressor

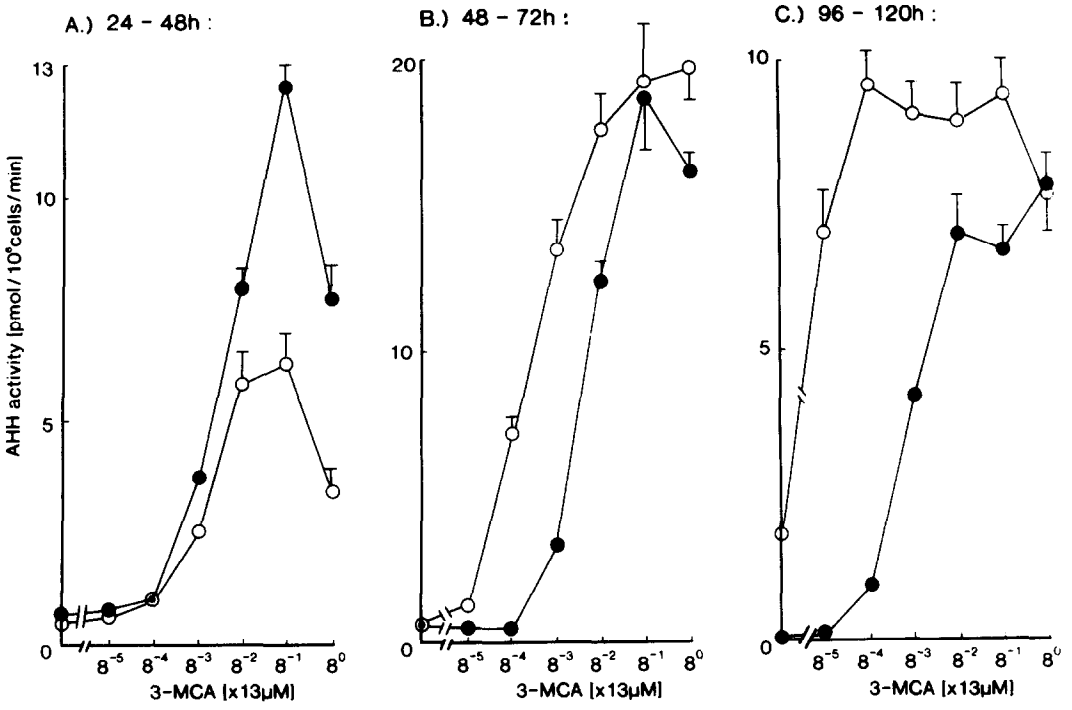

Fig. 5. AHH activity after treatment with 3-methylcholanthrene and/or cycloheximide and addition of actinomycin D. Hepatocytes at either day 1 (A), 2 (B) or 4 (C) were treated for 9 hr with (open circles) or without (closed circles) cycloheximide at 1 μg/mL and various amounts of 3-methylcholanthrene without medium change. Medium was then changed to a fresh one after several washings and actinomycin D at 2 μg/mL was added. AHH activity was determined 15 hr later. Each point represents the mean ± SD of data from four experiments.

Table 1. AHH induction by medium change and treatment with cycloheximide and actinomycin D

Culture period (hr)	Medium change*	Treatment CHI	Treatment ActD	AHH activity (pmol/10^6 cells/min)
24–48:				
	−	−	+	0.67 ± 0.07
	+	−	+	0.71 ± 0.05
	−	+	+	0.54 ± 0.04
	+	+	+	0.82 ± 0.10 ($P < 0.05$)
48–72:				
	−	−	+	0.61 ± 0.06
	+	−	+	0.49 ± 0.05
	−	+	+	0.60 ± 0.05
	+	+	+	7.24 ± 0.57 ($P < 0.001$)
96–120:				
	−	−	+	0.05 ± 0.01
	+	−	+	0.28 ± 0.10 ($P < 0.001$)
	−	+	+	1.84 ± 0.12 ($P < 0.001$)
	+	+	+	7.93 ± 0.50 ($P < 0.001$)

* Medium change was performed at the same time (+) as CHI addition or 24 hr before (−).

CHI, cycloheximide at 1 μg/mL for the initial 9 hr.

ActD, actinomycin D at 2 μg/mL for the last 15 hr.

AHH activities were measured at 48, 72 or 120 hr after the start of cultivation. Each value represents the mean ± SD of data from four experiments.

Table 2. Effect of medium change timing on AHH activity

Time of medium change* (hr)	Treatment CHI	Treatment ActD	AHH activity (pmol/10^6 cells/min)
120:			
	−	−	0.14 ± 0.02
	−	+	0.18 ± 0.03
	+	−	0.24 ± 0.06
	+	+	3.99 ± 0.61 ($P < 0.001$)
96:			
	−	−	0.17 ± 0.03
	−	+	0.11 ± 0.01 ($P < 0.02$)
	+	−	0.15 ± 0.03
	+	+	0.99 ± 0.11 ($P < 0.001$)
72:			
	−	−	0.15 ± 0.03
	−	+	0.09 ± 0.03
	+	−	0.14 ± 0.03
	+	+	0.73 ± 0.07 ($P < 0.001$)

* Medium was changed at 24-hr intervals, with the last change occurring as indicated in the Table.

Treatment with cycloheximide (CHI) at 1 μg/mL for 9 hr was started from 120 hr after seeding cells and then medium was changed to fresh one containing actinomycin D (ActD) at 2 μg/mL. AHH activity was determined 15 hr later. Each value represents the mean ± SD of data from four experiments.

protein [17–19] becomes down-regulated, or even that a gene controlling expression of the suppressor gene becomes extinguished, during the first 2 days in culture, thus making the cycloheximide effect more dramatic as a function of time in culture.

There have been several reports describing mouse *Cyp*1a-1 gene expression without exposure to so-called AHH inducers, for example after partial hepatectomy or during embryogenesis [15, 16]. Furthermore, the gene product could be detected in various tissues of man and non-treated rats by the reverse transcriptase-polymerase chain reaction method [14] and Puga *et al.* [30] recently observed derepression of *Cyp*1a-1 gene via transcription in mouse hepatoma cell lines by treatment with mevinolin, which is not a ligand for the *Ah* receptor and is an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, the rate-limiting step in cholesterol biosynthesis. These observations strongly suggest that the *Cyp*1a-1 gene product has some fundamental role under physiological conditions as well as being involved in the metabolism of carcinogenic aromatic hydrocarbons. Therefore, the possibility that a low level expression of *Cyp*1a-1 gene is necessary for maintaining mouse hepatocytes in culture is conceivable.

Superinducibility of the *Cyp*1a-1 gene by treatment with cycloheximide was first reported for a mouse hepatoma cell line by Whitlock and colleagues [17, 18] and has been observed in other established cell lines by Teifeld *et al.* [19]. These reports assumed the existence of a suppressor protein with a short life-span on the evidence that temporary inhibition of protein synthesis by cycloheximide enhanced both aromatic hydrocarbon-induced AHH activity and *Cyp*1a-1 gene transcript production. Enhanced expression of mRNA in association with cycloheximide treatment has also been observed for several other genes, for example *myc*, *fos* and *bcl*-2 [31, 32]. However, the proposed suppressor proteins have not been identified as yet. These observations were made in cells in culture and have not been made in the intact animal. In the present experiments, even if *Cyp*1a-1 gene transcripts were hardly detectable in untreated cells, the medium of which had been changed 24 hr before, they were manifested after cycloheximide treatment with increasing length of culture period. Since the character of hepatocytes shifts when they are transferred from the intact animal to primary culture, the lack of influence of cycloheximide treatment during the early phase of cultivation and the gradual elevation of responsiveness thereafter indicate that the regulatory mechanism might be brought into play after adaptation to culture conditions.

In addition to the observed cycloheximide-dependent presumed suppressor protein effects on AHH induction in cultured cells, a cAMP-dependent expression mechanism has been proposed by Yamazaki *et al.* [20], on the basis of results using a hamster kidney cell line. We found that cAMP-dependent regulation was not obvious immediately after seeding the mouse hepatocytes, but became conspicuous a few days later [33]. Thus, both cases suggest the need for further investigation into whether these regulation mechanisms really function *in vivo* or whether they are limited to isolated hepatocytes adapted to culture conditions.

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