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Aberrant CYP1A1 Induction: Discrepancy of CYP1A1 mRNA and Aryl Hydrocarbon Hydroxylase Activity in Mutant Cells of Mouse Hepatoma Line, Hepa-1

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We have isolated new benzo[*a*]pyrene-resistant clones, cl-21 and cl-32, of the mouse hepatoma line, Hepa-1. CYP1A1-dependent aryl hydrocarbon hydroxylase activity is not inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 3-methylcholanthrene in these two cell lines. However, mRNA of CYP1A1 is inducible in cl-21 and cl-32 cells, as in the wild-type cells, in spite of an undetectable level of cytosolic Ah receptor. The cl-21 cDNA of *Cyp1a-1* was found to have a single mutation leading to an amino acid substitution from Leu (118) to Arg (118). However, the CYP1A1 protein band was not detected on Western immunoblots. The cDNA of cl-32 was found to have a single mutation leading to an amino acid change from Arg (359) to Trp (359). The presence of the mature protein in cl-32 was confirmed by Western blot analysis. Somatic cell hybridization experiments demonstrated that the phenotype of cl-21 and cl-32 is recessive and that these clones belong to the same complementation group. These data suggest that there may be a non-Ah receptor-mediated mechanism of CYP1A1 induction.

Key words: Cytochrome P-450IA1 — Benzo[*a*]pyrene — Mouse hepatoma — Ah receptor

Cyp1a[1] represents one of eight known mammalian families in the P-450 gene superfamily.[2] The two mouse genes, Cyp1a-1 and Cyp1a-2, are induced by combustion products such as TCDD[2] and benzo[*a*]pyrene. Although the parent benzo[*a*]pyrene is not carcinogenic, the metabolites formed by the action of CYP1A1 are reactive intermediates that have been shown in many systems to be carcinogenic. It has been suggested that increased levels of CYP1A enzymes are highly correlated with environmental carcinogenesis.[3]

From mouse hepatoma Hepa-1 cell cultures treated with benzo[*a*]pyrene, it is possible to isolate benzo[*a*]-pyrene-resistant mutants. These mutant cells do not show induction of metabolic activity toward benzo[*a*]pyrene, and therefore do not generate the cytotoxic intermediates.[4] The availability of such mutant cells permits the use of both genetic and biochemical approaches in analyzing the induction mechanism of the carcinogen-metabolizing CYP1A1 enzyme.

In the present study, we examined the nucleotide sequence of CYP1A1 mRNA of two mutants. Both of these mutant cell lines have a CYP1A1 protein with a single amino acid differing from that of the Hepa-1 wild-type line. These mutants have no cytosolic Ah receptor activity, and lack nuclear XRE-binding activity, but they show a level of CYP1A1 mRNA equal to that of the wild type when treated with 3-MC or TCDD. The cell lines described herein should be valuable in assessing the contribution of CYP1A1 enzyme to the process of carcinogenesis. Our results imply that the mutant cells belong to a previously unidentified group or that there are other control factors involved in the CYP1A1 induction mechanism.

MATERIALS AND METHODS

Chemicals Cell culture medium was from Flow Laboratories Inc. (Irvine, Scotland, UK), fetal bovine serum from Hazleton Biologics Inc. (Lenexa, KS, USA), Geneticin (antibiotic G418) and mycophenolic acid from Sigma Chemical Company (St. Louis, MO, USA), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate from Boehringer Mannheim GmbH (Mannheim, Germany), and benzo[*ghi*]perylene from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). [1,6-H^3]2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (50 μg/ml, 20 Ci/mmol) and 2,3,7,8-tetrachlorodibenzofuran were purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA).

Cells The Hepa-1 cell line, derived from mouse hepatoma,[5] was kindly provided by Dr. Sogawa of Tohoku

[1] The recommendation of Nebert *et al.* (1991)[1] for notations of P450 proteins and gene families has been followed.

[2] Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHH, aryl hydrocarbon hydroxylase; 3-MC, 3-methylcholanthrene; XRE, xenobiotic responsive element; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; DMEM, Dulbecco's minimum essential medium; FCS, fetal calf serum; NRE, negative regulatory element.

University. The cells were maintained in DMEM containing 10% FCS, 100 μg/ml streptomycin and 100 units/ml penicillin G in humidified 95% air-5% CO_2 at 37°C. Benzo[*a*]pyrene-resistant Hepa-1 cells were isolated according to the protocol described by Hankinson[6] with minor modifications.

Enzyme assays The AHH activity assay,[7] protein assay,[8] and gel mobility assay[9] were performed as described previously.

Western blotting Microsomal proteins were prepared from about 10^8 cells as described in a previous report,[10] solubilized in SDS, resolved in 7.5% polyacrylamide gel and then transferred to a nitrocellulose sheet (Schleicher and Schuell GmbH, Dassel, Germany). Anti-CYP1A1 serum was prepared as described in a previous report.[10] Antigenic compounds reactive with anti-rat CYP1A1 serum were immunostained using a kit from Promega Biotech (Madison, WI, USA).

Northern blot hybridization The cells were quickly harvested and immediately frozen at −80°C until use. RNA was prepared by the guanidine thiocyanate method.[11] RNA was resolved on 1.1% formaldehyde agarose gel.[12] After electrophoresis, RNA was transferred to a nitrocellulose sheet (Schleicher and Schuell GmbH) by the capillary method.[13] Hybridization was performed as described in a previous report.[10]

PCR and subcloning Complementary DNA of the mutant cells was synthesized with AMV reverse transcriptase (Life Science Inc., St. Petersburg, FL, USA).[13] The oligonucleotide primers were 5′-CCACCTAGATCATGCCTTCC-3′ (P1S-1), 5′-GCCAACCTCTGCCATCACCT-3′ (P1AS-2), 5′-ATCTCGTCAGCAAACTTCAG-3′ (P1S-3), 5′-CTGTGTCTAGTTCCTCCTGG-3′ (P1AS-4), 5′-GTGGAGCCTCATGTACCTGG-3′ (P1S-5) and 5′-CTAAGCCTGAAGATGCTGAG-3′ (P1AS-6). The PCR was performed using 1/10 volume of cDNA reaction mixture and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer/Cetus, Emeryville, CA, USA) in 100 μl of reaction mixture.[14] PCR products were subcloned into the pCR1000 vector using a TA cloning kit (Invitrogen Corporation, San Diego, CA, USA). The DNA sequence was determined by the dideoxy termination method,[15] using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, OH, USA).

Ah receptor assay Specifically bound [^{3}H]TCDD in cytosol was assayed in terms of adsorption on hydroxylapatite as previously described by Houser *et al.*[16] and Gasiewicz and Neal.[17]

DNA transfection Supercoiled plasmid DNA was introduced into tissue culture cells (10 μg for 1×10^6 cells). Transformed cell lines harboring the *E. coli* gpt dominant marker gene, pSV3gpt,[18] were selected and cultured in XGPT medium containing DMEM supplemented with 0.2 μg/ml aminopterin, 5 μg/ml thymidine, 10 μg/ml xanthine, 25 μg/ml mycophenolic acid, 15 μg/ml hypoxanthine, and 2.3 μg/ml deoxycytidine.[19] Cell lines containing the selectable marker aminoglycol phosphotransferase, pWLneo,[20] were selected with the antibiotic G418 (100–800 μg/ml, depending on the cell line).

Transfection of Hepa-1 mutant cells was performed by the lipofection method[21] using Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, MD, USA).

Cell hybridization experiments In fusion experiments, one parent carried Eco-gpt gene (pSV3gpt) and the other parent carried neo^r gene (pWLneo). Cell fusion was done by the polyethylene glycol method described by Koyama *et al.*[22] The hybrids, grown in XGPT-G418 medium, were assayed for AHH by means of the benzo[*ghi*]-perylene sensitivity test.[23] The hybrid cells were inoculated at a concentration of 10^3 cells per 60-mm dish in medium containing 2 n*M* TCDD. Two days after plating, the medium was replaced with serum-free DMEM containing benzo[*ghi*]perylene. The cells were then incubated for 2 h, washed twice with serum-free medium, and incubated for 4 h in DMEM. After one wash with Hanks solution, the cells were illuminated for 10 min with near-ultraviolet light (UVP Inc., San Gabriel, CA, USA) at a flux of 1.78 mW/cm^2. Concentrations examined were 5, 10, 20, and 40 n*M* benzo[*ghi*]perylene. The cells were cultured for 10 days in DMEM-10% FCS and the colonies were stained with Giemsa solution.

RESULTS

Phenotypes of benzo[*a*]pyrene-resistant mutants A large number of clones observed in the benzo[*a*]pyrene selection dishes were isolated. Of these, 40 clones were then cultured in the presence of benzo[*a*]pyrene. All showed resistance to benzo[*a*]pyrene at the dose of 4 μg/ml. AHH activities were assayed in both non-induced and induced cultures of the 40 mutants. Induced cultures were treated with 1 μM 3-MC for 24 h. All 40 mutants had reduced AHH activities under both conditions. Wild-type cells showed 15-fold induction of AHH (73.0 $\pm$39.9 pmol/min/mg protein vs. non-induced AHH of 4.7$\pm$2.1 pmol/min/mg protein). On the other hand, the mean value of non-induced AHH of mutant cells was 4.9$\pm$7.3 pmol/min/mg protein and that of induced AHH was 7.5$\pm$8.0 pmol/min/mg protein.

Northern blot analysis In the course of this experiment, almost all the clones showed no hybridization signal or only very faint signals at the position of CYP1A1 (to be published), but we found that two specific clones, cl-21 and cl-32, exhibited increased levels of CYP1A1-mRNA (Fig. 1). Expression of β-actin served as a control in these experiments, and all RNA preparations were shown

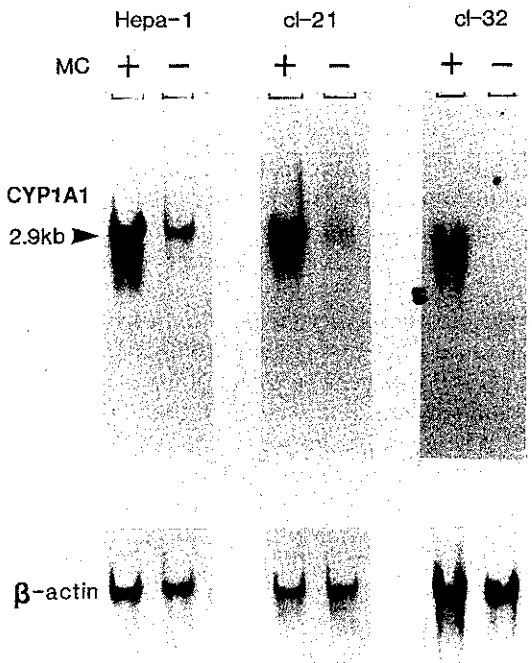

Fig. 1. Northern blot analysis. Hepa-1, cl-21 and cl-32 cells were treated with 3-MC (1 μM) for 24 h. Total RNA was isolated from the cells. Each lane contained 10 μg of total RNA. Rat Cyp1a-1 cDNA described in a previous report was used as a probe.

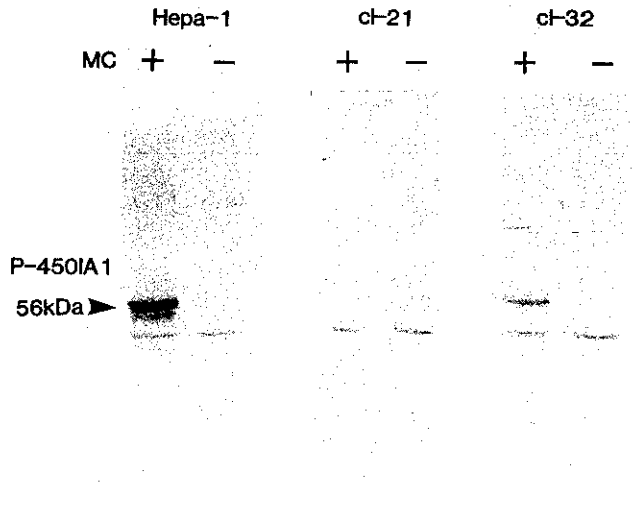

Fig. 2. Western immunoblot of CYP1A1 protein. Hepa-1, cl-21 and cl-32 cells were treated with 3-MC (1 μM) for 24 h. Following SDS-gel electrophoresis of microsomal proteins (20 μg of protein per lane) prepared from the cells, CYP1A1 protein was probed with anti-CYP1A1 serum. Molecular size is indicated in kilodaltons.

to hybridize equally with this actin probe (Fig. 1, bottom panel).

Western blot analysis Western blot analysis revealed that CYP1A1 protein of the wild-type lysate is identical to that in mouse liver microsomes (56,000 daltons), but the intensity of the band was faint in the mutant cl-32 cells, and the band was absent in the mutant cl-21 cells, as shown in Fig. 2.

***Cyp1a-1* cDNA sequences in the mutant cells** Comparisons of the sequence of *Cyp1a-1* cDNA from Hepa-1 wild type with those of the mutants (cl-21 and cl-32) revealed them to be completely identical, except for one mutation as noted below in each mutant. A single base difference between the wild type and cl-21 mutant, resulting in an amino acid change from Leu (118) to Arg (Fig. 3A) and a single-point mutation of cl-32 mutant, resulting in an amino acid change from Arg (359) to Trp (Fig. 3B), were detected.

To eliminate mis-incorporation of an incorrect base by *Taq* DNA polymerase, several independent PCRs were performed and the products were sequenced when a base substitution was found in the amplified DNA sequence.

Regulatory factors for CYP1A1 induction When intact cells are exposed to Ah receptor ligands, such as TCDD, there is initial binding to a cytosolic form of Ah receptor protein.[24] After a temperature-dependent transformation step,[25] the ligand-receptor complex becomes tightly associated with the cell nucleus and appears to acquire the ability to bind to specific DNA regions, thereby leading to enhanced transcription of mRNA coding for CYP1A1.

In order to clarify the changed step of the induction mechanism in the mutant cells, cytosolic Ah receptor was assayed by a method utilizing hydroxylapatite.[17] In these experiments, 1.0 nM [^{3}H]TCDD was used. The cytosol from wild-type cells, Hepa-1, showed high binding-site activity (70.3 fmol/mg protein). Mutant cells, however, showed low activity (cl-21, 1.9 fmol/mg protein and cl-32, 8.8 fmol/mg protein).

The DNA-binding factor specific for XRE was also assayed by the gel mobility shift method.[9] When the XRE-1 DNA fragment was used as a probe, a specific retarded band was observed only with the nuclear extract from 3-MC-treated Hepa-1 cells, as shown in Fig. 4. However, this band was extremely faint in the experiment in which the nuclear extract from the 3-MC-treated mutant cl-21 was used.

Cell hybridization studies To analyze the genetic basis for the lack of AHH activity in the mutant cells, cell hybridization experiments were performed. The fused cells were subjected to the single-step phototoxic procedure described previously.[23] In this procedure, only AHH-expressing cells can survive following selection

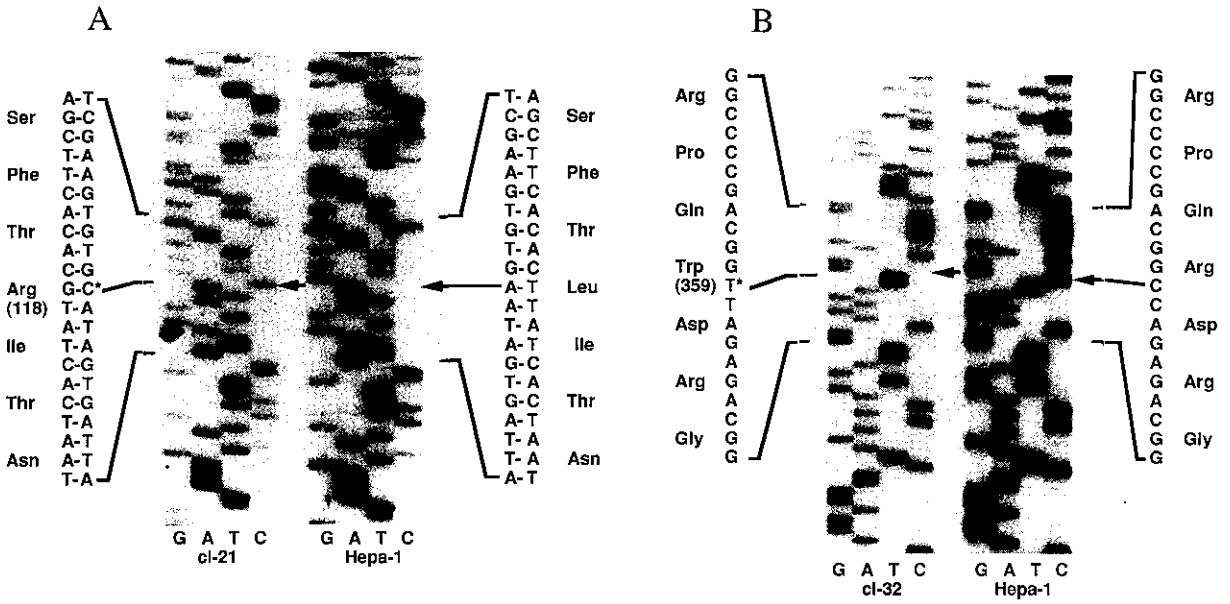

Fig. 3. Comparison of *Cyp1a-1* cDNA sequences in Hepa-1, cl-21 and cl-32 cells. A. The autoradiogram on the right exhibits the A-to-C substitution at position 118 of cl-21, and that on the left shows the corresponding part of a wild-type *Cyp1a-1* cDNA sequence. The sequencing shows anti-strand DNA. B. The autoradiogram on the right exhibits the C-to-T substitution at position 359 of cl-32 and that on the left shows the corresponding part of wild-type *Cyp1a-1* cDNA sequence. The asterisk denotes the mutated nucleotide.

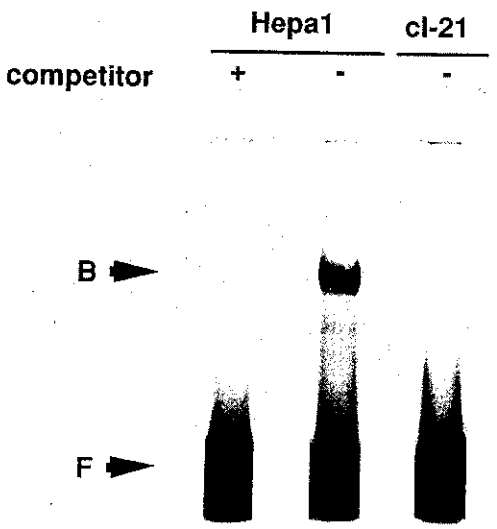

Fig. 4. Gel mobility shift assay. The nuclear extracts (4 μl) were prepared from Hepa-1 and from each mutant cell treated with 3-MC (1 μM) for 24 h. ^{32}P-Labeled XRE-1 (3×10^4 cpm) was used for the binding reaction. In the left lane, an excess amount of cold XRE-1 was added as a competitor.

because they are able to metabolize benzo[*ghi*]perylene to products that are no longer phototoxic. As shown in Fig. 5, there was a dose-dependent decrease in the number of AHH-deficient mutant cells surviving the benzo[*ghi*]perylene selection. When Hepa-1 was fused to either cl-21 or cl-32, the resulting hybrids were found to be inducible for AHH (Fig. 5A: only the result for cl-32 is shown). Therefore, the phenotype of the mutant cells is recessive. However, the fusion cell of cl-21 and cl-32 was not inducible for AHH (Fig. 5B). These results suggest that cl-21 and cl-32 belong to the same complementation group.

DISCUSSION

Many mutant cells derived from Hepa-1 cells have been reported by Hankinson's group[4, 6, 26] and Whitlock's group.[27] The cells were divided into 5 groups, that is, A, B, C, D and the dominant phenotype by Hankinson *et al.*[28] Mutations in the B, C and D genes all affect functioning of the Ah receptor. The dominant mutants were shown to synthesize a repressor of *Cyp1a-1* transcription.[29] The C gene was isolated and identified as the Ah receptor nuclear translocator, or Arnt gene,[30] which is required for translocation of Ah receptor-ligand complexes from the cytoplasm to the nucleus. The B gene is believed to be the Ah receptor gene.[31] The Ah receptor gene was isolated by Ema *et al.*[32] and Burbach *et al.*[33] The D^- mutant has a complex phenotype. It has a reduced level of Ah receptor, which is also diminished in

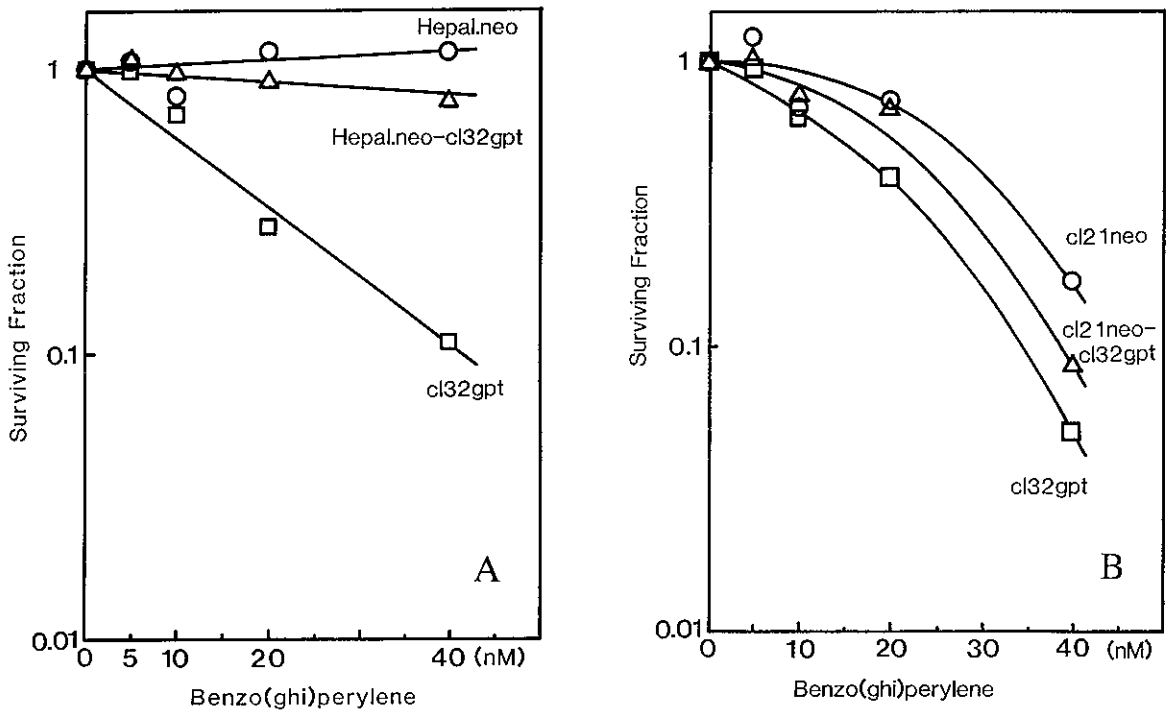

Fig. 5. Phototoxicity test of benzo[*ghi*]perylene against Hepa-1 cells and mutant cells. A. Complementation test of cl-32 against wild-type Hepa-1. The cells were inoculated at 10^3 cells per 60-mm dish in medium containing 2 n*M* TCDD. Two days after plating, the cells were cultured at various densities of benzo[*ghi*]perylene and illuminated with near-ultraviolet light. The cells were cultured for 10 days in DMEM-10% FCS and the colonies were counted. Hepa1.neo: Hepa-1 bearing pWLneo plasmid DNA; cl32gpt: cl-32 bearing Eco-gpt gene (pSV3gpt); Hepa1.neo-cl32gpt: hybrid cells of Hepa1.neo and cl32.gpt. B. Complementation test between cl-21 and cl-32. The treatment of cells was the same as described above. cl21neo: cl-21 bearing G418 resistance gene, pWLneo; cl21neo-cl32gpt: hybrid cells of cl-21.neo and cl-32.gpt.

its ability to translocate to the nucleus,[34] and TCDD does not induce CYP1A1-mRNA in these mutant cells. In the present study, we found that cl-21 and cl-32 mutant cells had no cytosolic Ah receptor activity, and lacked nuclear XRE-binding activity; however, they showed a level of CYP1A1-mRNA equal to that of the wild type when the cells were treated with 3-MC or TCDD. Therefore, our results imply that the mutant cells have unique characteristics and can be categorized as a new group, and that other control factors participate in the CYP1A1 induction mechanism.

The mutant cells showed a recessive phenotype and they were not able to complement each other (Fig. 5). Although TCDD did not induce AHH activity in either cl-21 or cl-32 cells, TCDD induced mRNA of CYP1A1 as potently as that of the wild type (Fig. 1). We therefore examined the nucleotide sequence of CYP1A1 mRNA of the two mutants. Both of the mutant-cell lines had CYP1A1 protein with a single amino acid differing from that of the Hepa-1 wild-type line. The amino acid change of Leu (118) to Arg in cl-21 may decrease the stability of the protein, because no protein band of CYP1A1 was detected by Western blot analysis (Fig. 2). The amino acid change of Arg (359) to Trp in cl-32 may change the activity of the CYP1A1 enzyme, resulting in low activity of AHH as described above. Because Arg (359) is located in the linker between helix J and helix K and the amino acids of this sequence are conserved in the CYP1A group,[35] the change of Arg to Trp may cause a drastic change of conformation of CYP1A1.

There are two possible mechanisms of CYP1A1 induction by an Ah-receptor-independent pathway. The first involves the existence of a repressor of *Cyp1a-1* transcription. Several groups[36, 37] have reported the superinduction of CYP1A1 transcription by cycloheximide in Hepa-1 cells. Their results suggested the existence of a labile protein(s) that acts to inhibit the function of the TCDD-responsive receptor/enhancer regulatory system via protein-protein interactions. A possible candidate for this type of protein factor is NRE-binding protein. Deletion analysis of the transcriptional activity of the 5′-flanking region revealed the negative element, that is NRE.[38] The

effect of NRE should be to repress the expression of the gene through interaction with a protein factor(s). If some component of our mutant cells became sensitive to TCDD-mediated perturbation due to a second mutation, CYP1A1 would be easily induced by TCDD-treatment.

The second possible mechanism is an induction of CYP1A1 by another signal transduction pathway. Omeprazole was previously shown to be an aryl hydrocarbon-like inducer of CYP1A1 and 1A2 in human hepatocytes in primary culture and *in vivo*.[39] However, Daujat *et al.* reported that omeprazole was not a ligand for the human liver Ah receptor by competition assay.[40] Therefore, omeprazole may have another effect on some signal transduction pathway, such as the protein kinase system. Thus, in hepatoma cells, there may be another pathway which induces the transcription of *Cyp1a-1*. For example, Okino *et al.*[41] reported that tetradecanoylphorbol acetate (TPA) inhibited the accumulation of TCDD-inducible liver CYP1A1-mRNA in the mouse. Several groups reported the involvement of protein kinase in the induction mechanism of CYP1A1.[42, 43]

In this report, we have demonstrated the loss of activity of cytosolic Ah receptor by the hydroxyapatite adsorption method, and the absence of nuclear Ah receptor binding with XRE sequence by gel mobility assay (Fig. 4). Preliminary studies showed that the mRNA of Ah receptor was detected in the wild-type cells, Hepa-1, but not in the mutant cells, cl-21 and cl-32, by Northern blot hybridization using polyA$^+$RNA (data not shown). However, it remains to be determined whether the Ah receptor gene is really not expressed in the mutant cells by a more sensitive method, such as the reverse-transcriptase PCR method. Furthermore, there is a possibility that Ah receptor gene has a mutation and this mutation may change the activity of Ah receptor in the mutant cells.

Exposure to TCDD produces a wide variety of toxic effects, including porphyria, liver damage, a wasting syndrome, epithelial hyperplasia and metaplasia, lymphoid involution, tumor promotion and teratogenesis.[44] There is a possibility that TCDD not only binds to the Ah receptor but also interacts with other components of signal transduction pathways, and one such component may have an altered function due to the mutation in our mutant cells.

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