0026-895X/01/6002-388–393$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 2
970/921713
Mol Pharmacol 60:388–393, 2001
Printed in U.S.A.

Loss of CYP1A1 Messenger RNA Expression Due to Nonsense-Mediated Decay

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Received March 20, 2001; accepted May 14, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Clones of the mouse hepatoma cell line Hepa1c1c7 (Hepa-1) with lesions in the *Cyp1a1* gene were isolated previously. A subset of these clones fails to express CYP1A1 mRNA even when treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which induces this mRNA in wild-type Hepa-1 cells. The current investigation sought an explanation for this phenotype in one of these clones, c33. Loss of mRNA expression in c33 was shown to be caused by mutational changes in the *Cyp1a1* gene rather than by its epigenetic silencing. No mutations were identified in the 5′ flanking region of the *Cyp1a1* gene, containing the promoter and dioxin-responsive enhancer sequences. A single nucleotide insertion occurred at nucleotide 418 in the coding region of one *Cyp1a1* allele, and a single nucleotide insertion occurred at nucleotide 465 in the other allele in c33. These sequence alterations were confirmed in the genomic DNA of the clone. Both insertions generate a premature termination codon at codon 172. This termination codon occurs in a position within the intron/exon structure of the *Cyp1a1* gene such that the encoded mRNA should be subject to "nonsense-mediated decay" (NMD). Inhibition of protein synthesis is known to reverse NMD. The protein synthesis inhibitors cycloheximide and puromycin fully restored CYP1A1 mRNA expression to c33 cells, supporting the notion that NMD degrades CYP1A1 mRNA in this strain. The mutations identified in the coding region of c33 provide an explanation, therefore, for its loss of both CYP1A1 enzymatic activity and inducible CYP1A1 mRNA expression.

The CYP1A1 protein possesses aryl hydrocarbon-hydroxylase (AHH) activity, which activates procarcinogens, including certain polycyclic aromatic hydrocarbons (PAHs). PAHs are widely distributed in the environment and are also present in cigarette smoke. CYP1A1-dependent metabolites of PAH bind DNA and form adducts, resulting in genetic damage and cancer. PAHs are not only substrates of CYP1A1, but also induce the enzyme. Induction occurs principally, if not exclusively, at the level of transcription. PAHs and certain polyhalogenated hydrocarbons, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin), bind the aryl hydrocarbon receptor (AHR), which then dimerizes the aryl hydrocarbon receptor nuclear translocator (ARNT) protein. AHR/ARNT dimers bind to xenobiotic-responsive elements (XREs) that contain the core sequence 5′-$^{T}/_{G}$NGCGTG-3′ located in the 5′ flanking region of the *Cyp1a1* gene and activate its transcription (Hankinson, 1995). We have used the mouse hepatoma cell line Hepa1c1c7 (Hepa-1), in which CYP1A1 is highly inducible, as a model system to study CYP1A1 and its regulation. We previously isolated clones of Hepa-1 cells defective in their induction of CYP1A1-dependent AHH by selecting for resistance to benzo[*a*]pyrene toxicity. Because clones were isolated after only a single exposure to benzo[*a*]pyrene, resistance was not acquired via stepwise multiple alterations (genetic or epigenetic). Clones in which loss of CYP1A1 inducibility is recessive were assigned to four complementation groups (i.e., genes) based on the results of somatic cell hybridization analysis. Using a mutant of the C group, we identified and cloned the human *Arnt* gene (Hoffman et al., 1991; Reyes et al., 1992). Complementation group D corresponds to the *Ahr* structural gene (Sun et al., 1997), and B mutants seem to be defective in their expression of the *trans*-acting factor required for expression of the *Ahr* gene (Zhang et al., 1996).

Mutants in complementation group A are defective in the *Cyp1a1* structural gene (Hankinson et al., 1985; Montisano and Hankinson, 1985; Kimura et al., 1987). Independently derived A mutants are heterogeneous with regard to expression of CYP1A1 mRNA and fall into three classes in this

This work was supported by National Institutes of Health/National Cancer Institute Grants R01-CA28868 and CA16042 (the latter to Jonsson Comprehensive Cancer Center).

ABBREVIATIONS: AHH, aryl hydrocarbon hydroxylase; PAH, polycyclic aromatic hydrocarbons; dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic-responsive elements; Hepa-1, Hepa1c1c7; RT-PCR, reverse transcriptase-polymerase chain reaction; kb, kilobase pair(s); NMD, nonsense-mediated decay; PTC, premature termination codon.

regard. Mutants of subgroup I retain some dioxin-inducible AHH activity and are also partially inducible for CYP1A1 mRNA. Subgroup III/IV mutants lack detectable dioxin-inducible AHH activity but are constitutive for CYP1A1 mRNA (i.e., they have high levels of CYP1A1 mRNA even when they are grown in the absence of dioxin). Point mutations were identified in the cloned CYP1A1 cDNAs from two subgroup III/IV mutants (Kimura et al., 1987). We proposed that the constitutive expression of CYP1A1 mRNA in subgroup III/IV mutants is caused by the accumulation of an endogenous inducer for the *Cyp1a1* gene that is normally subject to metabolism by CYP1A1 enzymatic activity (Hankinson et al., 1985), a model that has been proposed subsequently by other investigators (Weiß et al., 1996; Chang and Puga, 1998). The focus of this article is the group A, subgroup II mutants. These mutants were found to lack detectable dioxin-inducible AHH activity (less than 0.2% of the activity in equivalently treated Hepa-1 cells) and detectable dioxin-inducible CYP1A1 mRNA, as analyzed by RNA hybridization analysis (less than 3% of the level in equivalently treated Hepa-1 cells) (Hankinson et al., 1985). These mutants were of particular interest to us because their phenotype suggested that their analysis might provide novel insight into the mechanism of induction of the *Cyp1a1* gene. We describe the analysis of one of the group A, subgroup II mutants, c33.

Materials and Methods

Cell Culture Procedures. The mouse hepatoma cell line Hepa-1 and its derivatives were maintained in nucleoside-free α-minimal essential medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum in a 5% CO_2 incubator at 37°C. The group A, subgroup II mutants c33, c38, and c40 had been isolated from Hepa-1 cells treated with ICR-191G, a frame-shift mutagen, and c41 had been isolated from Hepa-1 cells treated with the alkylating agent ethanyl methanesulfonate before their selection in benzo-[*a*]pyrene (Hankinson, 1981). For 5-aza-2′-deoxycytidine treatment, c33 cells were treated with a fresh stock solution of the compound (Sigma Chemical, St. Louis, MO) at a final concentration of 1 μM plus 5 nM dioxin for 2 days, and the cells were then pooled for total RNA isolation. A semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of CYP1A1 mRNA levels was then performed with the use of the same primers used for CYP1A1 cDNA sequencing, as described below. A segment of glyceraldehyde 3-phosphate dehydrogenase was also amplified with RT-PCR as a control by use of 5′ primer 5′ACCACAGTCCATGCCATCAC3′ and 3′primer 5′TCCACCACCCTGTTGCTGTA3′ (CLONTECH, Palo Alto, CA). Transfections were performed using the GenePORTER reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's protocol. The reverse selection was performed as described previously (Hankinson, 1991).

Isolation of Genomic DNA and RNA. Genomic DNA was isolated from Hepa-1 cells and its derivatives using QIAamp Blood Kit (QIAGEN, Valencia, CA). Total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, CA).

PCR and RT-PCR Amplification. Primers and cycle conditions for PCR and RT-PCR are listed in Table 1. The positions of the PCR primers are illustrated in Fig. 1. PCR amplifications, including cycle sequencing, were performed by use of the PTC-200 DNA Engine (MJ Research, Watertown, MA). The mouse *Cyp1a1* gene was isolated from genomic DNA of wild-type Hepa-1 cells and the c33 mutant by PCR amplification using the Expand Long-Template PCR System 1 (Roche Molecular Biochemicals, Mannheim, Germany). A segment of c33 genomic DNA encompassing the single nucleotide insertions identified in this work was also PCR-amplified using the Expand High-Fidelity PCR System (Roche). Dimethyl sulfoxide (10%) was included in these PCR reactions. CYP1A1 cDNA containing the complete coding sequence was amplified by RT-PCR. Reverse transcription was performed with 4 μg of total RNA using SuperScript RNase H^- reverse transcriptase (Invitrogen) and oligo(dT) as a primer. Amplification of the first-strand cDNA was then carried out using the Expand High-Fidelity PCR System (Roche). Before sequencing, PCR products were purified using the PCR purification kit (QIAGEN) or were first separated by agarose gel electrophoresis and then purified using the Gel Extraction Kit (QIAGEN). The primers for PCR amplification of the CYP1A1 cDNA were designed to include *Bam*HI and *Kpn*I sites. The cDNA product was digested with *Bam*HI and *Kpn*I and cloned into plasmid pTarget (Promega, Madison, WI).

Cycle Sequencing. Cycle sequencing was performed using the BigDye Terminator and AmpliTaq DNA polymerase FS (Applied Biosystems, Foster City, CA) on an Applied Biosystems ABI 377 automated sequencer. DNA was sequenced from both directions by use of the primer walking strategy, with neighboring primers spaced approximately 400 bp apart. The sequence we obtained for 5′ upstream region of *Cyp1a1* (−1525 to +6) has been submitted to GenBank. The accession number is AF210905.

Northern Blot Analysis. Cells were grown to 95 to 100% confluence and then treated with either 10 μg/ml cycloheximide or 30 μg/ml puromycin. One hour later, dioxin was added, to a final concentration of 10 nM, and the cells were then harvested after another 6 hours. Total RNA was isolated with the use of CLONTECH's NucleoSpin RNA II kit. Total RNA was loaded (4 μg per lane), electrophoresed in a 1% agarose gel, and transferred and then UV cross-linked to a nitrocellulose membrane. The membrane was hybridized sequentially to a 700-bp fragment from the 3′ untranslated region of the mouse *Cyp1a1* cDNA and then to a cDNA corresponding to the constitutively expressed gene, CHOb, each of which was labeled with ^{32}P by random priming (Hankinson et al., 1985; Zhang et al., 1996). The membranes were then subjected to radioautography, and radioactive bands were quantified with the use of a PhosphorImager (445 SI; Molecular Dynamics, Sunnyvale, CA) administered by the UCLA Biological Chemistry Imaging Facility.

Results

Inability to Restore CYP1A1 mRNA Expression to c33 by Treatment with 5-Azacytidine. Takahashi et al. (1998) demonstrated that the lack of CYP1A1 inducibility in

TABLE 1
A list of PCR primers for amplifying the following regions of the *Cyplal* gene and cycling conditions

Amplified Region		Primers	Cycling Conditions
Whole gene	ON31:	AAA TGT GGA CAC ACG CTG	94°C × 15 s → 58°C × 30 s
	ON32:	GGG TGT GAA TGT GGC TTA AC	→ 68°C × 8 min, 35 cycles
Promoter	ON22:	TGT AAC TAG AGT GGG AGG TG	94°C × 15 s → 55°C × 30 s
	ON23:	GCT CCA AGA ACT ACC ACC	→ 72°C × 90 s, 30 cycles
cDNA	ON38:	CGG GAT CCT TAC AGC CCA AGC AGC	94°C × 15 s → 60°C × 30 s
	ON39:	GGG GTA CCC AGA GCA CTC TTC AGG AG	→ 68°C × 2 min, 35 cycles
Genomic region containing insertions	5′P:	TAA TGG CAA GAG CAT GAC	94°C × 15 s → 55°C × 30 s
	3′P:	TCC AGA GCC AGT AAC CTC	→ 68°C × 45 s, 30 cycles

a rabbit lung cell line is caused by methylation of cytosine residues in the CpG sites within the XRE sequences in the 5′ flanking region of the gene and that CYP1A1 inducibility could be restored by treatment of the cells with 5′-aza-2′-deoxycytidine, which leads to demethylation of 5-methylcytosine residues in DNA (Takahashi et al., 1998). We believed that the lack of CYP1A1 mRNA inducibility in c33 might also be caused by de novo methylation of cytosine residues in the *Cyp1a1* gene. To test this possibility, we treated c33 with 5′-aza-2′-deoxycytidine and then analyzed for dioxin-inducible CYP1A1 mRNA expression by an extremely sensitive semiquantitative RT-PCR procedure. 5-Azacytidine treatment did not lead to an increase in CYP1A1 mRNA expression in the c33 strain, thus counting against the notion that its diminished mRNA expression is caused by methylation of the *Cyp1a1* gene (Fig. 2).

Rescue of the Mutant Phenotype by Transfection with the *Cyp1a1* Gene Isolated from Wild-Type Hepa-1 Cells but not with the Gene Isolated from the Mutant Itself. Further experiments were performed to determine whether 1) epigenetic changes, including processes such as silencing via chromatin modification [which may be responsible for loss of CYP1A1 inducibility in our B complementation group of Hepa-1 cell variants (Zhang et al., 1996)] and cytosine methylation, or 2) genetic changes were responsible for loss of CYP1A1 mRNA inducibility in c33. These experiments used our previously described "reverse selection" procedure (Van Gurp and Hankinson, 1983), which is capable of selecting for rare AHH-positive (i.e., CYP1A1 inducible) cells in the presence of a vast excess of AHH-negative (i.e., CYP1A1 noninducible) cells. From both Hepa-1 cells and the c33 strain, we PCR-amplified a 7.5-kb genomic fragment that encompasses all exons and introns of the gene plus a 1.5-kb segment from the 5′ flanking region that contains all its promoter elements and most of the flanking XREs. The uncloned PCR products were then transfected into c33 cells, and the cells were subjected to our reverse selection to test for rescue of the mutant phenotype. An important advantage of using PCR is that this process converts 5′-methylcytosine residues to cytosines. An advantage of using uncloned, rather than cloned, PCR products for transfection is that potential confounding effects of any polymerase errors that may occur are eliminated. Whereas the *Cyp1a1* segment from wild-type Hepa-1 cells was capable of rescuing the c33 mutant phenotype, the *Cyp1a1* segment from c33 cells was inactive in this assay (Table 2). This experiment therefore demonstrates that sequence alternation(s) in the *Cyp1a1* gene of the c33 strain must be responsible for its noninducibility phenotype and therefore that the phenotype of this strain originated by a mutational, rather than an epigenetic, mechanism.

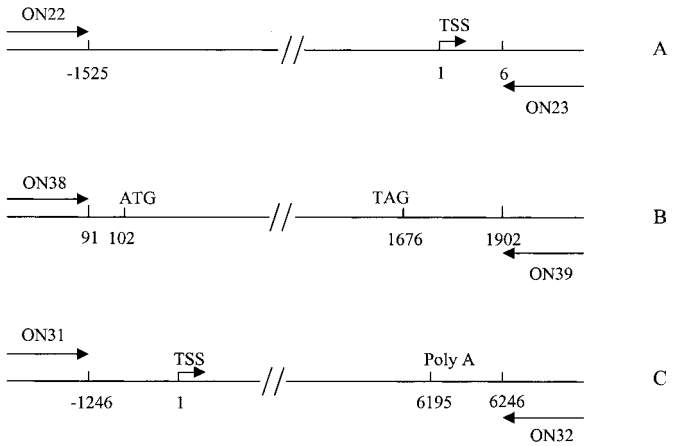

Fig. 1. A diagram of the PCR or RT-PCR strategy for amplifying the different regions of the *Cyp1a1* gene. A, 5′ regulatory region; B, cDNA; C, whole gene. (Not all to the same scale.) TSS; transcriptional start site.

Sequence Analysis of the 5′ Flanking Region of the *Cyp1a1* Gene. A 1.5-kb genomic fragment of the *Cyp1a1* gene covering the transcriptional initiation site, all promoter sequences, and most XREs was amplified from Hepa-1 and c33 (and also from three other subgroup II strains). Uncloned PCR products were sequenced in both directions using primers spaced approximately 400 bp apart. This sequencing strategy permits identification of heterozygous mutations (Anttila et al., 2000; and see under *Discussion*). c33 exhibited neither homozygous nor heterozygous changes relative to the parental Hepa-1 strain in the 5′ flanking region of the *Cyp1a1* gene (this was also true of the other group A, subgroup II strains); therefore, the c33 phenotype is not ascribable to mutations in this region. All five strains contained 23 sequence differences relative to the corresponding sequence contained in GenBank.

Sequence Analysis of cDNAs for the *Cyp1a1* Gene. To test whether mutations occur in the coding region or splice junctions of the *Cyp1a1* gene, we amplified cDNA containing the complete coding region of the gene from strain c33 by RT-PCR. A single band of the size predicted for the full-length cDNA was generated after 35 cycles of PCR amplification of the reverse-transcription product. No abnormally sized products, indicative of splicing variants, were detected. A second round of PCR amplification was then performed to obtain sufficient DNA for sequence analysis. Sequencing of the sense strand of the uncloned RT-PCR product revealed an unambiguous sequencing pattern, except in the segment between nucleotides 418 and 462, where many ambiguous bases were indicated (Fig. 3). Sequencing the antisense strand of the same segment revealed the same pattern (Fig. 3). This suggested that one allele has a base insertion (or deletion) at the beginning of the ambiguous sequence and that the other allele has a base insertion (or deletion) at the end of the ambiguous sequence. To further identify the nature of these genetic changes, we cloned the PCR products and sequenced the segment of interest in six clones (Fig. 4). Two clones had a G insertion at base 418 (the A in the start codon is numbered 1), and the other four clones had a C insertion at base 465. To exclude the possibility that these insertions might be errors generated by RT-PCR, we PCR-amplified this region from genomic DNA and sequenced the

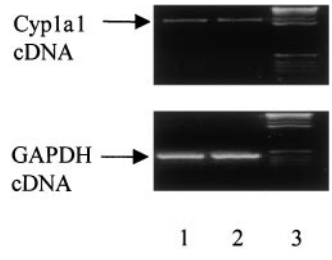

Fig. 2. RT-PCR analysis of CYP1A1 mRNA. Total RNA was isolated from (1) c33 cells treated with 5-aza-2′-deoxycytidine and (2) c33 cells without treatment. A segment of glyceraldehyde 3-phosphate dehydrogenase cDNA was also amplified as a control. Lane 3 is a 1-kb DNA marker.

PCR products from both directions. A segment of ambiguous bases started and ended at the same nucleotides as those in the cDNA. This segment was flanked by unambiguous sequence. Thus, each allele contains a single base insertion. The insertion at base 418 generates a nonsense codon at position 172 of this allele. The insertion at base 465 in the other allele also generates a nonsense codon at position 172.

Restoration of CYP1A1 mRNA Expression in c33 cells by Inhibition of Protein Synthesis. The nonsense codon at position 172 is more than 55 nucleotides upstream of the last exon/intron splicing junction of the *Cyp1a1* gene and would therefore be predicted to confer instability to the encoded mRNA via nonsense-mediated decay (NMD) (Zhang et al., 1998). We therefore investigated whether NMD might explain the lack of CYP1A1 mRNA expression in c33 cells. NMD is known to be blocked by protein-synthesis inhibitors (Carter et al., 1995) and we therefore studied the effects of two such inhibitors, cycloheximide and puromycin, at concentrations that are known to inhibit protein synthesis by 95 to 97% (Israel and Whitlock, 1983; Israel et al., 1985). Both protein-synthesis inhibitors dramatically increased CYP1A1 mRNA levels in dioxin-treated c33 cells (Fig. 5, lanes 1–6). Levels were also increased in c33 cells not treated with dioxin, which was most probably caused by the accumulation of an (endogenous) inducer by these AHH-deficient cells (Hankinson et al., 1985). Neither inhibitor restored CYP1A1 mRNA expression to the ARNT-deficient mutant c4 (Fig. 5, lanes 7–12). Both protein-synthesis inhibitors also increased the levels of CYP1A1 mRNA in dioxin-treated Hepa-1 cells to some degree (Fig. 5, lanes 13–18). This poorly understood "superinduction" phenomenon has been observed previously (Israel and Whitlock, 1983; Israel et al., 1985). Messenger RNA levels were quantified by PhosphorImaging analysis of the blot, and the ratio of the counts in the CYP1A1 band to those in the CHOb band was determined for each lane. This ratio for c33 cells treated with dioxin plus cycloheximide (15.0, lane 4) was similar to that for equivalently treated Hepa-1 cells (13.2, lane 16). The ratio for c33 cells treated with dioxin plus puromycin (8.4, lane 6) was also similar to that for equivalently treated Hepa-1 cells (8.2, lane 18). Thus, both protein synthesis inhibitors restored dioxin-induced CYP1A1 mRNA expression in c33 cells to fully wild-type levels. These results therefore lend very strong support to the notion that lack of CYP1A1 mRNA in c33 cells is caused by nonsense-mediated decay.

TABLE 2

Reverse selection of c33 cells transfected with the wild-type or c33 *Cyplal* gene

5×10^5 c33 cells with or without 400 Hepa-1 cells were inoculated per 100-mm dish (two dishes per treatment). One day later, they were subjected to the reverse selection. Fourteen days later, the dishes were stained and colonies were counted.

Cells	DNA Transfected	Number of Clones Survived Reverse Selection/ 5×10^5 cells
c33	None	3
c33 + WT (400 cells)	None	175
c33	c33 *Cyplal* gene	2
c33	WT *Cyplal* gene	86

WT, wild-type.

Discussion

We were very interested in the group A, subgroup II mutants because their lack of CYP1A1 mRNA expression suggested that they might be mutated in a segment of the *Cyp1a1* gene required for transcription. We therefore studied one of these mutants, c33, in detail. We found that c33 contains different single base-pair insertions in the coding region of each *Cyp1a1* allele. Both insertions generate a premature nonsense mutation at codon 172. The proteins encoded by the defective mRNAs would be 139 (encoded by allele 1) and 154 (by allele 2) amino acids, respectively, which correspond to approximately one fourth the length of the wild-type CYP1A1 protein.

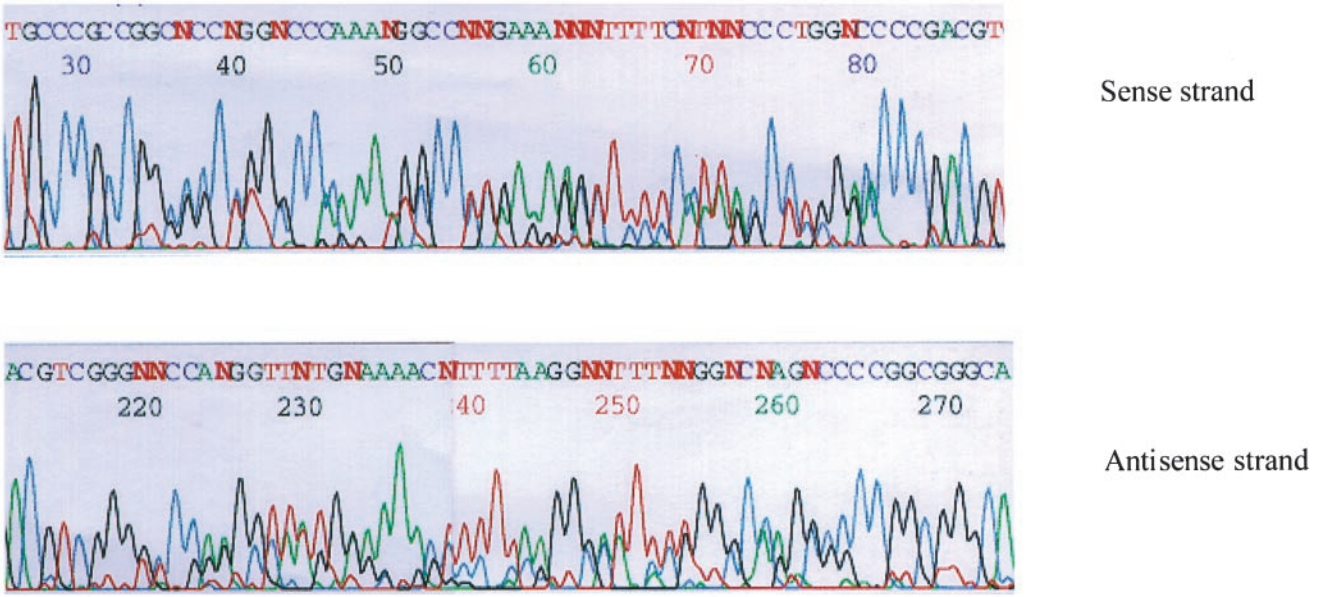

Fig. 3. Sequencing profile of a segment of the uncloned CYP1A1 cDNA. The sense and antisense strands of the CYP1A1 cDNA were directly sequenced without cloning. The segment containing many ambiguous bases flanked by clear sequences is shown. In the sense strand, the ambiguous region starts immediately after 5′ flanking sequence "TGCCCGCCGG" and ends just before 3′ flanking sequence "CCCGACGT". In the antisense strand, the ambiguous region starts right after 5′ flanking sequence "ACGTCGGG" and ends just before 3′ flanking sequence "CCGGCGGGCA".

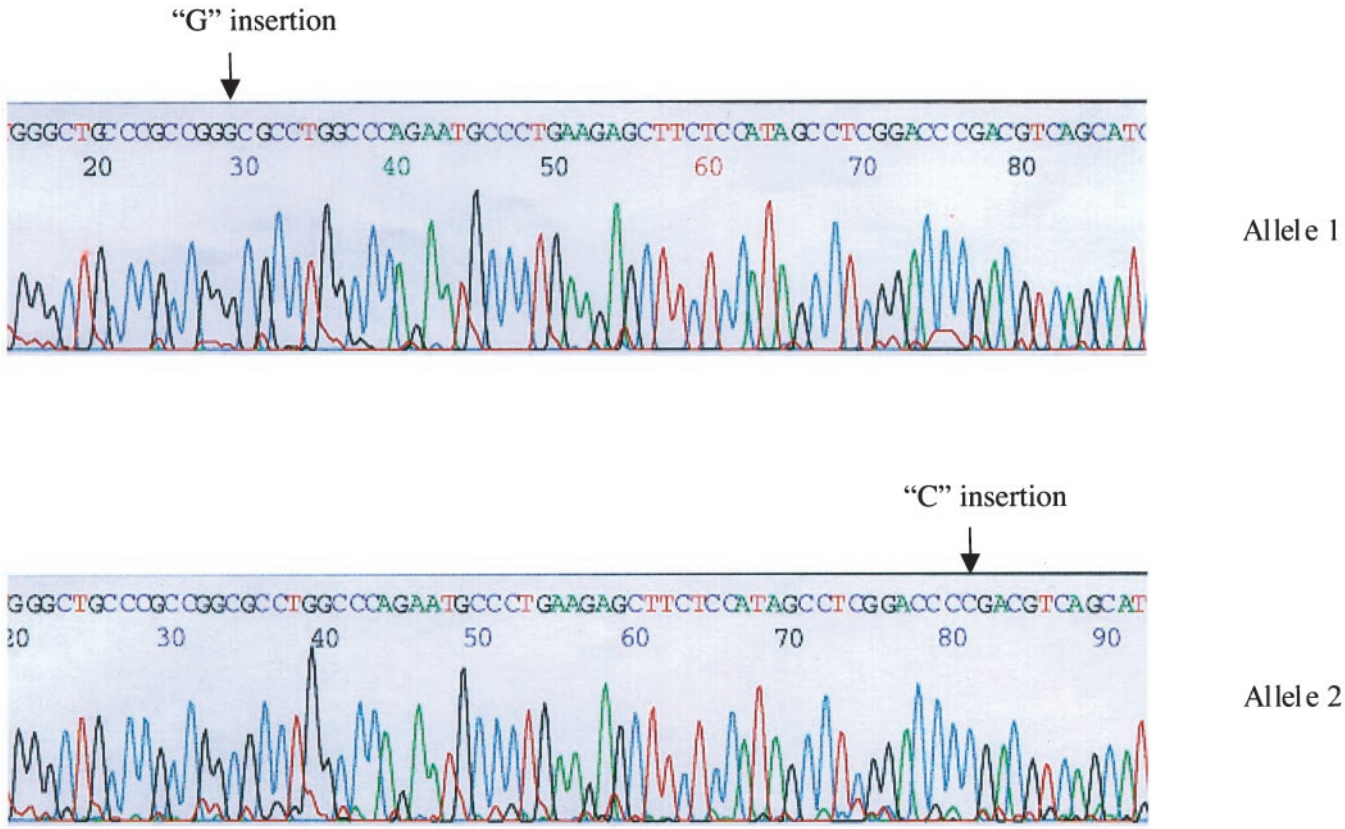

Fig. 4. Sequencing profile of CYP1A1 cDNA clones. CYP1A1 cDNA was RT-PCR amplified and six clones were sequenced. The sense sequence of the segment shown in Fig. 2 is shown here.

c33 (and other subgroup II mutants) possess less than 3% of the CYP1A1 mRNA levels of the parental wild-type Hepa-1 cells after dioxin treatment. Both of the above single-base insertions generate the same premature termination codon (PTC). Cells have evolved a surveillance mechanism, NMD, for rapidly degrading PTC-containing mRNA. NMD has been observed in all eukaryotic systems, ranging from yeast to humans (Culbertson, 1999; Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999). The biological importance of NMD is likely to be to prevent accumulation of carboxyl-terminal truncated proteins, which could act in a dominant negative fashion over their normal counterparts. Only those mRNAs with PTCs located at least 50 to 55 nucleotides upstream of the last exon/intron splicing junction are subjected to NMD (Zhang et al., 1998). In the case of c33, the PTCs occur far upstream from the last splicing junction, thus satisfying the requirement for NMD. Our observation that two protein synthesis inhibitors that have different mechanisms of action restore CYP1A1 mRNA expression to c33 strongly supports the notion that lack of CYP1A1 mRNA in these cells is caused by NMD. (Cycloheximide inhibits the peptidyl transferase step of protein synthesis, whereas puromycin causes premature peptide chain termination by acting as an analog of charged tRNA.) Our results therefore demonstrate that both aspects of the phenotype of the c33 mutant [1) its lack of CYP1A1 enzymatic activity and 2) its noninducibility for CYP1A1 mRNA] can be fully explained by mutations in the coding region of the *Cyp1a1* gene. CYP1A1 mRNA seems to be an inherently short-lived mRNA, with a half-life of 2.4 h in human hepatoma cells (Lekas et al., 2000).

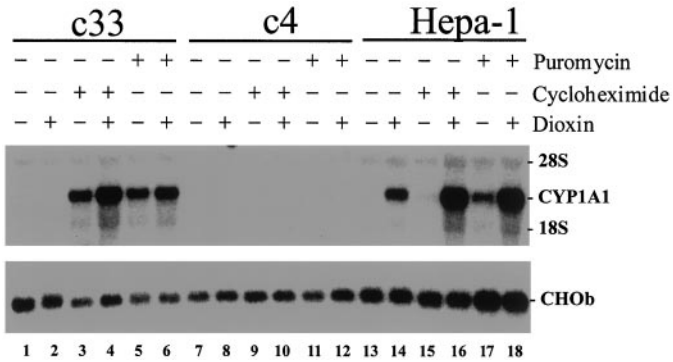

Fig. 5. CYP1A1 mRNA expression in c33 cells treated with protein-synthesis inhibitors. c33 (lanes 1–6), c4 (lanes 7–12), and Hepa-1 (lanes 13–18) cells were treated with cycloheximide (10 μg/ml) or puromycin (30 μg/ml). One hour later, 10 nM dioxin was added (as indicated), and the cells were harvested 6 hours later. Total RNA was analyzed by Northern blot analysis for CYP1A1 mRNA and CHOb mRNA (used as a loading control). The locations of the 18S and 28S ribosomal RNAs are shown.

The three other group A, subgroup II mutants that we studied, which were also shown not to contain mutations in the upstream regulatory region of the *Cyp1a1* gene, may also owe their phenotypes to NMD, generated either by frame-shift mutations or base-substitution mutations leading to nonsense codons. Like c33, five of the other seven subgroup II mutants we isolated had been obtained from cultures treated with the frame-shift mutagen ICR-191 (Hankinson et al., 1985).

The single base-pair insertions we identified in the CYP1A1 cDNA provide an opportunity to appraise the sen-

sitivity of sequencing uncloned RT-PCR products for identifying heterozygous mutations. The stretch of nucleotide sequence between the insertions at nucleotides 418 and 465 contains 33 nucleotide substitutions relative to the wild-type sequence. Although not all the resulting heterozygous nucleotide positions are recognized by the (proprietary) Applied Biosystems "Base Caller" sequencing software we used (such heterozygous sites are indicated by "N" in Fig. 3), by eye we easily identified 30 of 33 of the heterozygous sites in the sense strand sequence and 31 of 33 in the antisense strand sequence, indicating that our overall sensitivity for detecting heterozygous mutations is 92%, even if only one strand is sequenced. If both strands are sequenced, the sensitivity should exceed 99%. As discussed elsewhere, our protocol calls for us to routinely scan uncloned cDNA or genomic DNA sequences by eye to detect heterozygous mutations or polymorphisms (Anttila et al., 2000).

Acknowledgments

We thank Anna Asanbaeva for assistance and Steven Rivera for advice.

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