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MOLECULAR CLONING OF THE HUMAN AH RECEPTOR GENE PROMOTER†

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A λ phage clone containing a promoter region of the human Ah receptor gene was isolated. This clone spanned 13.8 kb and contained the 1st exon, the sequence of which completely matched the reported Ah receptor cDNA. Using RNase protection assay and primer extension analysis, the transcription initiation sites were determined to be 643 and 615 bp upstream of the translational initiation codon ATG. This promoter did not contain a TATA box, while multiple GC boxes were present close to the determined transcription initiation sites. Comparison of the 5' flank sequence of the human Ah receptor with its murine equivalent showed several well conserved regions, containing binding sites for known transcription factors, such as Sp1. The promoter activity was confirmed by transient transfection of chimeric constructs of the Ah receptor gene and reporter gene luciferase into hepatoma HepG2 cells.

Cytochrome P4501A1 (CYP1A1) catalyzes many environmental carcinogens, such as benzo(a)pyrene and 3-methylcholanthrene, to their ultimate DNA-binding carcinogenic forms. Expression of this enzyme is induced drastically by its inducers, such as arylhydrocarbons (Ah) and halogenated aromatic hydrocarbons represented by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)(1). The mechanism of induction by these compounds occurs at the transcriptional level and is mediated by the Ah receptor (AhR) (TCDD receptor), a ubiquitous intracellular 100 kDa protein possessing a basic-helix-loop-helix motif and a PAS domain (2). Exposure of ligands to the AhR modulates release of hsp90 from the AhR (3). After hetero-dimerization with its partner protein, AhR nuclear translocator (Arnt), the complex regulates the transcription of the target genes (4,5).

†The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession number: D31708.

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Recently, we partially determined the structure of the human AhR gene and developed a quantitative RT-PCR analysis. With this method, AhR mRNA concentration in human tissues has been shown to be highly variable; some of the highest AhR-expressing tissues in human were the lung, kidney, and blood (6). Moreover, Dolwick et al. reported high expression of AhR mRNA in the lung and placenta using Northern blot analysis (7). Considering human CYP1A1 has been detected in the lung, lymphocytes and placenta (8), AhR can be a dominant determinating factor for the expression of the CYP1A1 gene. In order to understand the biological roles of AhR in specific tissues and the regulation of its expression, it is necessary to characterize the regulatory elements of the AhR gene. Recent study by Schmidt et al. showed the cloning and characterization of the murine AhR gene, providing a clue to investigate the AhR gene regulation (9). In this study, we cloned and analyzed the promoter region of the human AhR gene to give a further information to unwrap the molecular mechanisms of AhR regulation in human.

Materials and Methods

Materials- Human lung cDNA library and human genomic library were obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). Restriction enzymes, T4 polynucleotide kinase, Klenow fragment, ligation kit, T7 RNA polymerase, and AMV reverse transcriptase were purchased from TAKARA Shuzo (Tokyo, Japan). Taq DNA polymerase was obtained from Perkin-Elmer and Pfu DNA polymerase was from Stratagene. [α-^{32}P]dCTP(3,000 Ci/mmol), [α-^{32}P]UTP(800Ci/mmol), [α-^{35}S]dATP(1,000Ci/mmol) and [γ-^{32}P]ATP(3,000Ci/mmol) were from Amersham. Lipofectin was from GIBCO BRL.

Oligonucleotides- Oligonucleotides were synthesized using the Applied Biosystems DNA synthesizer Model 392 (Applied Biosystems). The sequence of oligonucleotides used in this study was as follows: AHR28, GCA GTG GTC CCA GCC TAC AC; AHR29, GTT TTC TGC ACC GGC TTC CGC; AHG12, ACG CTC TCG GAA CAG AGC GTC GAC GGG ACT; AHGPE, ACA CGG CTG CCC CTG TTC TGA GCT GGC GGT.

Library Screening- Initially, a 600 bp fragment of human Ah receptor cDNA was generated by the PCR method using Pfu DNA polymerase. The resulting fragment was radiolabeled by multiprime labeling using [α-^{32}P]dCTP (3,000Ci/mmol). It was then used as a probe for screening the human lung cDNA library. Hybridization was conducted in 5xSSC containing 0.5%(v/v) SDS, 100 μg/ml of denatured herring sperm DNA, 5 mM of EDTA, and 1xDenhardt's solution at 65°C over night. Nylon membranes were washed 3 times in 2xSSC/0.1% SDS at room temperature, twice in 0.5xSSC/0.1% SDS at 65°C for 30 min each, and twice in 0.1xSSC/0.1%SDS at 65°C for 30 min. Phage DNA was prepared by LamdaSorb adsorbent (Promega) and subcloned into pGEM7Zf(+) (Promega). Sequencing was carried out by Sequenase Ver.2 kit (USB) or by AutoRead sequencing kit followed by A.L.F. DNA Sequencer (Pharmacia LKB, Uppsala, Sweden). In order to screen the human genomic library, a 240 bp human AhR cDNA fragment spanning -83 to +156 bp (relative to the translational initiation codon ATG) prepared from the isolated λ clone L13-1 was radiolabeled by a multiprime labeling kit (Amersham) and [α-^{32}P]dCTP (3,000 Ci/mmol). Hybridization and washing conditions were the same as described above. Four positive λ clones were isolated and subjected to PCR exon mapping using Taq DNA polymerase with primers AHR28 and AHR29 for detection of the 1st exon.

Cell Culture and Total RNA Preparation- Human hepatoma HepG2 cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10%

bovine calf serum. Total RNA was prepared by the acid guanidium/thiocyanate/phenol/chloroform method (10).

Primer Extension Analysis- For primer extension, the oligonucleotides, AHGPE and AHG12, were phosphorylated by T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol). The radiolabeled primer was hybridized with different amounts of HepG2 total RNA or yeast tRNA as a control. The complex was incubated with 50 units of AMV reverse transcriptase for 2 hrs at 42°C. Extended DNA fragments were analyzed on 6% acrylamide/7M urea gel. The same oligonucleotide was used with cloned genomic DNA to generate a sequencing ladder as molecular weight markers.

RNase Protection Assay- A 280 bp ApaI-SacI (-768 to -490) fragment of the AhR gene was subcloned into pGEM11Zf(+) (Promega). The resulting plasmid was linealized by digestion at the XbaI site. *In vitro* transcription was conducted using T7 RNA polymerase (TAKARA Shuzo, Tokyo, Japan) in the presence of [α-^{32}P]UTP (800Ci/mmol) as described (10). The radiolabeled transcripts were applied onto a NAP-10 column (Amersham) and the peak fractions were collected and ethanol precipitated in the presence of 2M NH_4OAc. The purified transcripts were hybridized with various amounts of HepG2 total RNA or 30 μg of yeast tRNA in 80 % formamide at 45°C over night. The RNA-RNA complex was digested with 10 μg/ml of RNase A and 0.5 μg/ml of RNase T1 at 30°C for 30 min. The digested products were resolved on a 6% acrylamide/7 M urea gel. As a size marker, known lengths of transcripts were prepared as described above.

Promoter Activity Analysis- A 3.4 kb EcoRI-HindIII fragment of the human AhR gene was subcloned into pGEM7Zf(+). A 2.5 kb XbaI-NaeI fragment of the resulting plasmid was prepared and subcloned into pSP72 vector (Promega) at the XbaI and PvuII sites, respectively. A 2.5 kb XhoI-XhoI fragment of the plasmid was ligated to the reporter gene plasmid pGL2 basic (Promega) at the XhoI site to construct pGLAHG25. The direction of inserts were determined by sequencing pGLAHG25. A 5' deletion construct (pGLAHG06) was prepared by digestion of pGLAHG25 with BssHII and NheI followed by blunt-ending and self-ligation. The pGL2-Control vector driven by SV40 promoter/enhancer (Promega) was used as a control. All the plasmids used for the transfection experiments were prepared by a QIAGEN column (QIAGEN Inc., Chatsworth, CA). The constructs were transfected into HepG2 cells by a lipid-mediated procedure (12). Luciferase activity was assayed in 240 μl of reaction mixture containing 40 μl of cell extracts using the Luciferase Assay System (Promega) in a Titertek Luminoskan luminometer. The integrated light units were normalized against the protein concentration of cell extracts.

Computer Analysis of 5' flank of AhR Gene- In order to search putative *cis*-acting elements in the 5' flank of the AhR gene, the sequence was analyzed by TFD (transcription factors database)(13). Comparison of the human AhR gene with murine AhR was carried out by the method of Gotoh (14).

Results and Discussion

Screening of the human AhR gene- The 240 bp fragment from the human AhR cDNA clone was used as a probe to screen a human placenta genomic library. Four positive clones were isolated from 1.2 x10^6 plaques. Taking the high identity of human AhR cDNA and the murine counterpart in the N-terminus region (5) into consideration, we synthesized a pair of primers AHR28 and AHR29 to detect the region corresponding to the putative 1st exon of the human AhR gene by PCR. A PCR analysis of the isolated λ clones showed that only a clone named λ AHG6 contained the putative 1st exon, while the other three clones contained the 2nd exon, but not the 1st exon (data not shown). In order to focus on the promoter region of human

AhR, a further study was carried out using the λ AHG6 clone. The inserts of λ AHG6 were subcloned into pGEM7Zf(+), and PCR mapping of these subclones was carried out as above. A subclone pAHGEH34 containing a 3.4 kb EcoRI-HindIII fragment of λ AHG6 was found to possess the 1st exon and was subjected to complete sequencing. The pAHGEH34 coded the 1st exon and the location of the 1st exon-1st intron junction of the human AhR gene was identical to that of the murine AhR gene (9). The coding region sequence was completely identical to that reported for human AhR cDNA (7), while the 5' noncoding region possessed several nucleotide differences between the cDNA sequence and the genomic sequence determined by this study. These minor differences might have occurred either by a sequence misreading of the GC rich region, a mis-incorporation of nucleotides during the synthesis of cDNA or polymorphism in this region.

Determination of transcription initiation site(s) of the human Ah receptor gene- To determine the transcription initiation site(s) of the human AhR gene, we conducted an initial investigation by means of RNase protection assay. A labeled riboprobe prepared from the ApaI-SacI fragment (-768 to -490) was hybridized with HepG2 total RNA followed by digestion with RNase A and RNase T1. As shown in Fig.1A, one major protected band was observed around a size of 130 bp. The intensity of the band was increased proportionally with the amounts of added total RNA. In addition, there was a minor protected band around 160 bp, which also showed an increase relative to the amounts of RNA, though the control lane showed some protected bands close to that position. To determine the exact location of the transcription initiation site(s) of the AhR gene, we next performed primer extension analysis using radiolabeled primer AHGPE and HepG2 total RNA. As shown in Fig.1B, two major extended products were detected at -643 and -615 bp, both of which correspond to the transcription sites determined by RNase protection assay. The intensity of these products was also proportional to the amount of added HepG2 total RNA. When an additional primer extension experiment using primer AHG12 was performed, primer extended products corresponding to the upstream site were also obtained (data not shown). Taking together these results, we concluded that the transcription initiation sites of the human AhR gene were located 643 and 615 bp upstream of the translation initiation codon ATG. The 5'-untranslated region of the human AhR gene is much longer than that of the murine AhR gene, which has been reported to possess multiple transcription initiation sites and a 5'-noncoding region with a length that varies from 371 to 426 bp (and 20% of those sites may be located further upstream) (9). This difference in the lengths might partially contribute to the difference in the mRNA size of these two genes; human AhR is 6.6 kb in length and murine AhR is 5.4 or 5.0 kb (7,15).

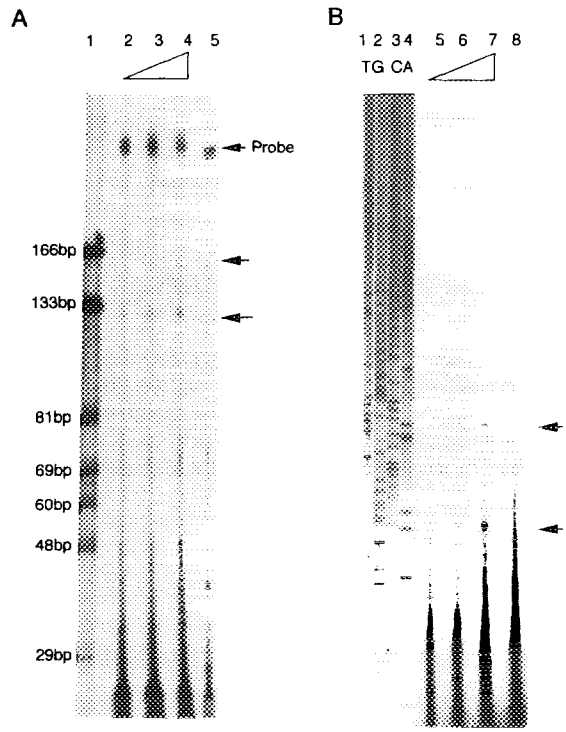

Fig.1. Determination of the transcription initiation site of human AhR. (A) RNase protection assay of AhR mRNA. The radiolabeled riboprobe was hybridized with 5.6 μg (lane 2),or 16.5 μg (lane 3), or 56.0 μg (lane 4) of HepG2 total RNA or 30 μg of yeast tRNA (lane 5). After digestion with RNases T1 and A, the protected fragments were subjected to 6% polyacrylamide/ 7 M urea gel electrophoresis. Appropriate riboprobes of known sizes were used as molecular weight markers. The arrows indicate the protected fragments. (B) Primer extension analysis of human AhR. The radiolabeled primer AHGPE was hybridized with 5 μg (lane 5), 15 μg (lane 6), or 50 μg (lane 7) of HepG2 total RNA or 50 μg of yeast tRNA(lane 8) and the extended products were applied onto 6% polyacrylamide/7 M urea gel. Arrows indicate the initiation sites. Sequencing ladders were used as size markers.

Analysis of the human AhR promoter activity- To confirm the promoter activity, the 5' flank together with the 5' noncoding sequence of the human AhR gene was ligated to the reporter gene, luciferase, and the resulting constructs were transfected into HepG2 cells (Fig.2). The luciferase activity of cell extracts transfected with pGLAHG25 was comparable to that with control plasmid pGL2-Control vector driven by the SV40 promoter/enhancer, indicating that the 2.5 kb EcoRI-NacI fragment of AhR gene is capable of acting as a functional promoter in HepG2 cells. Moreover, an 1.9 kb deletion of pGLAHG25 from the 5' end (pGLAHG06) caused approximately a 23-fold reduction of luciferase activity, suggesting the presence of some *cis*-acting elements in the deleted region. Additional deletion and point mutation analysis will be required to determine the organization of transcription factors involved in the initiation complex of the AhR gene.

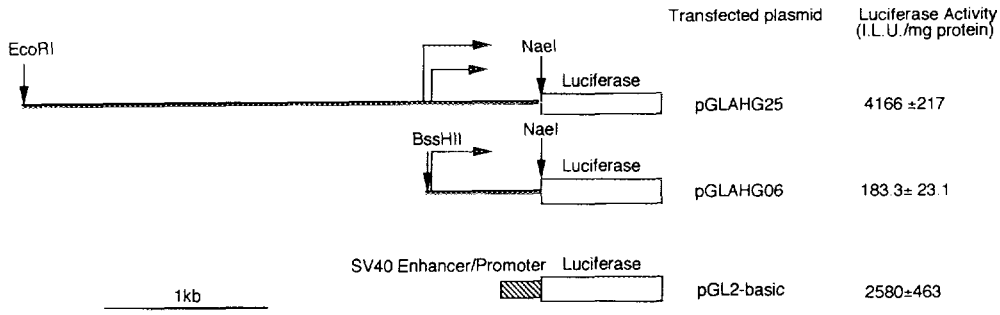

Fig.2. Promoter activity of the human AhR gene. Human hepatoma HepG2 cells were transfected with 2 μg of the indicated plasmids by lipofection, and cell extracts were prepared as described in Materials and Methods. The integrated luciferase activity was normalized to the protein concentration of the cell extracts. Each value is the average of the results of three independent experiments and standard deviations from the means are presented.

Comparison of the human and murine AhR genes- Since biologically important elements within the proximal promoter regions are generally conserved among species, we compared the human and murine AhR gene sequences (9) (Fig.3). Both of these genes contained a

Fig.3. Comparison of human and mouse AhR gene sequences. Sequences of the human and mouse AhR gene (9) were aligned by the program of Gotoh. The nucleotide positions were numbered to the translational initiation codon. The asterisk represents a matched nucleotide. A bar represents a gap in the alignment. The locations of GC boxes, E-box, and binding sites for CREB are underlined. Positions of transcription initiation sites are shown by arrows.

highly GC rich region in the 5' flank as well as the 5' untranslated region. One of the major transcription initiation sites of human and murine AhR was located at the corresponding site within a conserved region (Fig.3). In addition, certain stretches showed a well conserved sequence among these genes. The most notable of these were multiple GC boxes, which are binding sites for transcription factor Sp1, adjacent to the transcription initiation sites (Fig.3). The multiple GC boxes are found in a number of TATA-less promoters, including housekeeping genes (16). Apart from the GC boxes, binding motifs for the transcription factors, CRE and E-box, were also conserved among the human and murine receptors. Interestingly, CRE-binding proteins have been reported to be involved in a placenta-specific expression of the human glycoprotein hormone α-subunit gene (17,18). In contranst, two other binding motifs with the sequences TGGTAAA and GAAAACAGGT which have been pointed out as placenta-specific *cis*-elements in murine AhR gene (9) were not conserved in human AhR gene. On the other hand, the E-box is recognized by Myc and USF, both of which belong to the basic-helix-loop-helix family (19). It would be of great interest to identify the organization of transcription factors in relation to the biological function of AhR.

The mechanism of the regulation of CYP1A1 gene expression is now well understood at the molecular level, but little is known about its dominant regulator AhR, which regulates a number of different genes other than CYP1A1. Actually, it has been suggested that AhR participates in the biochemical and toxicological effects of TCDD, such as tumor promotion, thymus involution, teratogenesis, and hepatic porphyria (20). Therefore, analysis of the regulatory elements of the AhR gene would provide insights for understanding the tissue-specific biological function of AhR.

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