ISOLATION OF THE HUMAN AH RECEPTOR PROMOTER AND IDENTIFICATION OF GENETIC VARIATIONS

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Introduction

The Ah receptor (AhR) is a ligand-activated transcription factor belonging to the bHLH(basic helix-loop-helix)-PAS protein family of transcription factors. It mediates biological and toxic responses of e. g. halogenated aromatic hydrocarbons and certain indole derivatives [Schrenk et al., 1997]. After ligand binding, the AhR heterodimerizes with the bHLH protein Arnt (Ah receptor nuclear translocator), forming a complex which is translocated into the nucleus where it can bind to xenobiotic regulatory elements in enhancers of target genes (e. g. several drug-metabolizing enzyme genes). In many mammalian and in several nonmammalian vertebrate species the AhR has been identified. In humans it has been found in various tissues such as lung, liver, kidney, placenta and tonsils [Bock, 1993; Rowlands and Gustafsson, 1997].
Although much is known about the biochemical and molecular mechanisms of AhR action, little is known about the control of the expression of the *AhR* gene itself. In the present study we first aimed at the identification of regions important for constitutive *AhR* gene expression. For this purpose we constructed reporter vectors including 2.7 kb of the AhR 5'-flanking region and unidirectionally deleted promoter fragments. In addition we looked for polymorphisms within the 5'-flanking region of the *AhR* gene in 94 healthy Caucasian volunteers using PCR-SSCP (single-strand conformational polymorphism) analysis.

Methods and Materials

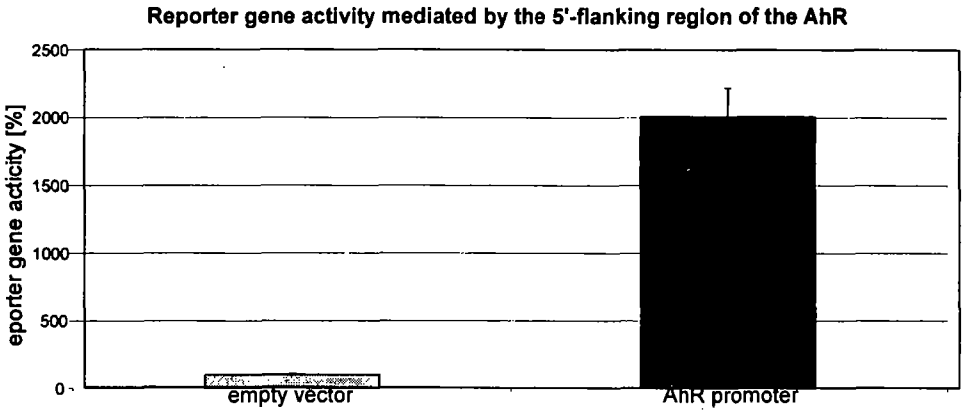
A PCR-fragment spanning 2.7 kb of the human *AhR* gene 5'-flanking region was amplified with the Advantage®-GC Genomic Polymerase Mix (CLONTECH, Heidelberg, Germany). After ligation in a TA vector (PCR II, Invitrogen, Groningen, The Netherlands) it was subcloned in a luciferase reporter vector (pGL3-Enhancer, Promega, Heidelberg, Germany) using KpnI and XhoI sites in the multicloning region. The correct identity of the insert was confirmed by sequencing using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia, Freiburg, Germany).
Unidirectional deleted inserts were constructed with the erase a base® system (Promega, Heidelberg, Germany) according to the instructions of the manufacturer.
HepG2 cells cultured in DMEM including 20 % FBS and 1 % penicillin / streptomycin were co-transfected with reporter gene constructs and the control reporter gene plasmid pRL-SV40 (Promega, Heidelberg, Germany), containing the renilla luciferase gene, using the calcium phosphate co-precipitation method as described previously [Kauffmann and Schrenk, 1998]. One day before transfection, confluent HepG2 cells had been trypsinized and seeded at a density of about 40.000 cells / cm^2 on collagenated 60 mm petri dishes. For transfection of each plate 1 µg reporter gene

construct, 0.1 µg of the pRL-SV40 plasmid were used. After 30 minutes the precipitate was added slowly to the HepG2 culture dishes (600 µl per plate). After 4 h medium was changed and cells were incubated for additional 48 h.
Reporter gene assays were performed using the Dual luciferase™ system reporter assay (Promega; Heidelberg, Germany). Cells were washed twice with PBS and lyzed in PLB (passive lysis buffer). Cell homogenates were analyzed luminometrically (Lumat LB 9507, Berthold; Wildberg, Germany) according to the instructions of the manufacturer. After background correction (activities in untreated cells) relative reporter gene activities were calculated by dividing the firefly luciferase activity (reporter gene) by the renilla luciferase activity (control gene).

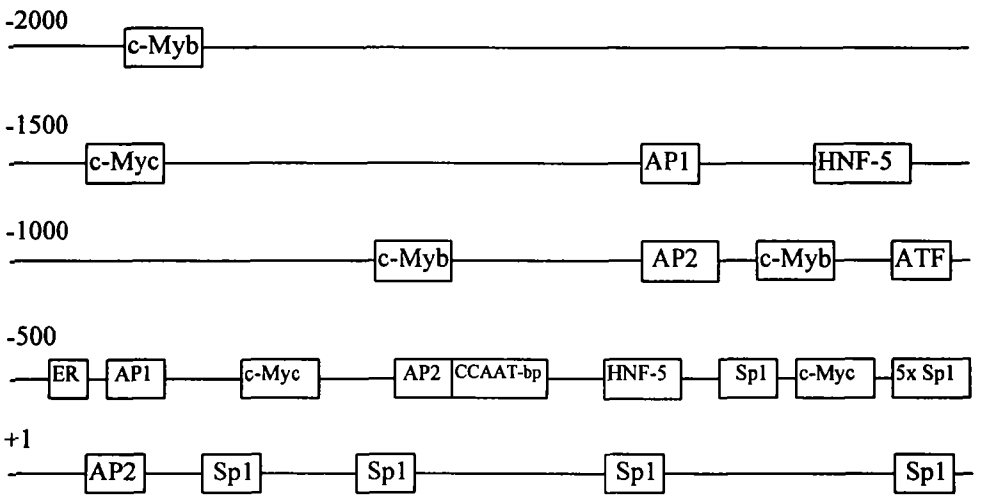
Screening for polymorphisms was performed with DNA of blood samples collected from a cohort of 94 healthy Caucasian volunteers. Isolation of mononuclear cells from peripheral blood was performed using the VACUTAINER®CPT™ system (BECTON DICKINSON, Franklin Lakes, USA), and DNA was isolated with QIAamp Blood Maxi Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturers.
In order to identify variations within the 5'-flanking region of the *AhR*-gene, a polymerase chain reaction-based single strand conformation polymorphism (PCR-SSCP) analysis was carried out. This method is based on differences in electrophoretic mobility of single-stranded nucleic acids resulting in conformational differences.
First, the 5'-flanking region was subdivided into overlapping PCR fragments of approximately 300 bp which were amplified. PCR products were heat denaturated and separated via native PAGE (polyacrylamide-gel electrophoresis) at 4°C [Jaeckel et al. 1998]. DNAs were visualized using a silver staining procedure and recorded with a gel documentation system (EAGLE EYE, Stratagene, Heidelberg, Germany).

Results and Discussion

First we constructed a luciferase reporter gene construct including 2.7 kb of the 5'-flanking region of the AhR. Reporter gene assays demonstrated that this part of the AhR promoter strongly activates the reporter gene (about 20-fold) compared with the empty reporter vector.



Using the HUSAR program TRANSFAC we identified several putative binding sites for transcription factors in the flanking region [Wingender et al., 1997]. These factors include e. g. AP1, AP2, ATF, ER, HNF-5, c-Myb, c-Myc, Sp1, and CCAAT binding proteins. Using the unidirectionally deleted constructs we intend to determine which of these putative binding sites are important for the basal expression of the AhR gene.
A factor which might regulate the expression of the human AhR is Sp1, as it is described to be involved in the regulation of the murine AhR [Fitzgerald et al., 1998].



By performing PCR-SSCP analysis we identified for the first time three polymorphisms in the 5'-flanking region. These polymorphisms include two point mutations at about bp –500 and +150 of the 5'-flanking region. Interestingly, one of these mutations is localized adjacent to a putative ATF-site and might therefore influence its binding profile. The third polymorphic site comprises a polyG sequence in which the number of guanosine residues varies between 8 and 11. This genetic variation is localized near a putative Sp1 site and might therefore also be able to influence regulatory processes.

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