SCREENING FOR GENETIC VARIABILITY OF THE HUMAN AHR (AH RECEPTOR) GENE 5'-FLANKING REGION AND THE HUMAN ARNT (AH RECEPTOR NUCLEAR TRANSLOCATOR) GENE BY SSCP ANALYSIS AND DISTRIBUTION STUDY OF CYP1A2 PHENOTYPES IN A COHORT OF HEALTHY CAUCASIAN VOLUNTEERS

Julia Scheel, Judith Racky, Ragna Hussong, Hans-Joachim Schmitz and Dieter Schrenk

Department of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, D-67663 Kaiserslautern, Germany

Introduction

The aryl hydrocarbon receptor (AHR) has been identified in many mammalian and in a number of nonmammalian vertebrate species. After ligand binding, the AHR translocates to the nucleus where it forms a dimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). Both AHR and ARNT belong to the basic helix-loop-helix/PER ARNT SIM (bHLH/PAS) family of proteins. Through interaction with xenobiotic-responsive elements (XREs) in the 5'-flanking region of responsive genes like cytochrome P450 (CYP) 1A1 and 1A2 the whole complex acts as a nuclear transcription factor. [Denison et al. 1988].

Furthermore, ARNT acts as a heterodimerization partner of various structural related proteins and thus participates in many regulatory processes. Together with hypoxia-inducible factor (HIF-) 1α it is involved in the cellular response to low oxygen levels. In addition ARNT heterodimerizes with a human homolog of the Drosophila *SIM* gene which is located in the region of chromosome 21 critical for Down's Syndrome. [Rowlands and Gustafsson 1997].

Interindividual differences in the level and inducibility of CYP1A2 activity were observed. [Butler et al. 1992; Schrenk et al. 1998]. Polymorphisms of the *CYP1A2* gene have not been reported, thus these differences might be due to genetic polymorphisms of AHR or ARNT. However, a genetic polymorphism in exon 10 of the human AHR gene was reported to have no impact on CYP1A2 inducibility [Kawajiri et al. 1995]. Alternative explanations could be genetic polymorphisms in the *ARNT* gene or in the 5'-flanking region of the *AHR* or *ARNT* gene. Within the known sequence of the 5'-flanking region of the human *AHR* gene potential binding sites for different transcription factors have been identified, but their involvement in the control of *AHR* gene expression is not yet clear.

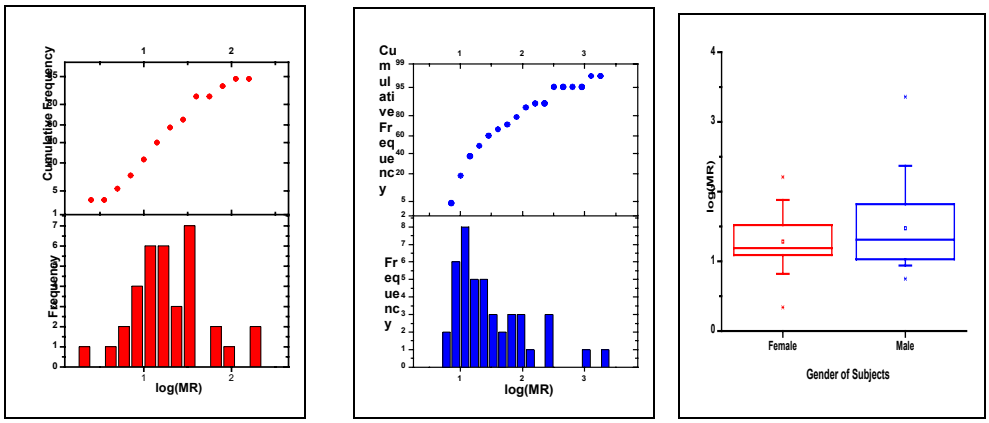
In this study we report on the single-stranded conformational polymorphism (SSCP) analysis of some DNA fragments in the 5'-flanking region of the *AHR* and *ARNT* gene and of some exons of the *ARNT* gene in 94 healthy Caucasian volunteers. Part of the 5'-flanking region of *ARNT* gene was sequenced and possible regulatory elements were determined by homology comparison. A distribution analysis of a urinary ratio of caffeine metabolites (MR_c) representative of CYP1A2 activity was done in parallel.

Materials and Methods

Blood samples were collected from a cohort of volunteers comprising 94 individuals (40 females, 54 males). Urine was collected from 84 individuals of the same cohort (47 males, 37 females) for 6h after intake of caffeine. Isolation of mononuclear cells from peripheral blood was performed

using the VACUTAINER®CPT™ system (BECTON DICKINSON, Franklin Lakes, USA) according to the instructions of the manufacturer.
DNA was isolated with QIAamp Blood Maxi Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer.
In order to identify polymorphisms within the 5'-flanking region of the *AHR*- and *ARNT* gene and in three exons of the *ARNT* gene, a polymerase chain reaction-based single strand conformation polymorphism (PCR-SSCP) analysis was performed. The 5'-flanking regions were subdivided into overlapping PCR fragments of approximately 300 bp. Exons were amplified by constructing primers (20mers) aimed directly at the exon-intron boundaries.
The PCR-SSCP method is based on differences in electrophoretic mobility of single-stranded nucleic acids resulting in conformational differences. The DNA sequence of interest is first amplified by PCR and then heat-denatured DNA fragments are separated via native PAGE. [Jaeckel et al. 1998] 5 µl PCR product was incubated with 7.5 µl denaturing buffer at 97°C for 10 minutes. After that the samples were placed on ice immediatly for 5 minutes. 10 µl of the mixture were loaded onto the pre-cooled gel and run at 4°C.
To investigate the genomic structure of the *ARNT* gene combined strategies were persued, a) a genome walking procedure with GenomeWalker™Kit (CLONTECH, Heidelberg, Germany), and cloning of PCR products with the TOPO TA cloning system (INVITROGEN, Groningen, The Netherlands), sequencing of the clones, b) test of similarity with exon-intron boundaries of the mouse *ARNT* gene [Wang et al. 1998] by constructing primers and performing PCR and c) PCR screening of a human BAC library (GENOME SYSTEMS, St. Louis, USA), subcloning *Hind*III restriction fragments of a positive BAC clone in pBluescript II KS (STRATAGENE, La Jolla, USA) and sequencing of the subclones. Sequencing was performed either with a LI-COR 4000L (MWG-BIOTECH, Ebersberg, Germany) using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (AMERSHAM, Cleveland, USA) or with an ABI Prism TM analyse system 377 (PERKIN ELMER, Weiterstadt, Germany).
Clones containig parts of the human *ARNT* gene were identified by hybridization with an *ARNT* cDNA probe.
For CYP1A2 phenotyping urine was analysed for representative caffeine metabolites (AFMU=5-acetylamino-6-formylamino-3-methyluracil, 1U=1-methyluric acid, 1X=1-methylxanthine, and 17U=17-methyluric acid) by HPLC (BECKMAN, Munich, Germany) on an ODS RP column as described [Schrenk et al. 1998].
Distribution of a CYP1A2-dependent MR_C =[AFMU+1X+1U]/17U was analysed using cumulative distribution (probit) analysis. Separate box plots were established for males and females.
Sequence comparison was done by GAP (HUSAR software package).

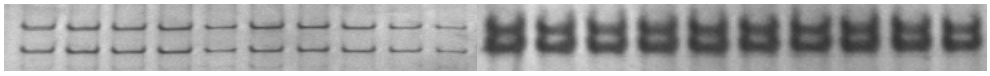
Results and Discussion
Frequency analysis and probit plots (Fig. 1 A and B) show slightly distorted log-normal distributions which do not rule out the possibility of polymodality. Earlier investigations showed a bimodal distribution [Schrenk et al. 1998]. This could be an evidence for polymorphisms in CYP1A2 activity. The box plot (Fig. 1 C) shows a slightly lower CYP1A2 activity in females compared to males.



A **B** **C**

Fig. 1 Distribution of CYP1A2-related metabolic ratios among female (n=35) and male (n=43) nonsmokers. **A** and **B**: Frequency and probit plots. **C**: Box plot. Because the number of smokers in the cohort was too small only the frequency distributions of female and male nonsmokers were calculated.

Neither in the *AHR* 5'-flanking region nor in the *ARNT* gene any polymorphism could be detected by SSCP analysis in the fragments investigated so far.



A **B**

Fig. 2 Example of SSCP analysis of genomic DNA from 10 individuals of *AHR* 5'-flanking region, 305 bp fragment (**A**), *ARNT* 5'-flanking region 290 bp fragment (**B**).

Sequencing of about 500 bp of the human *ARNT* 5'-flanking region revealed several possible regulatory elements. Some are shown in Fig. 3. In contrast to the mouse sequence a TATA-box was found whereas two GC boxes were detected in the mouse upstream region. The CRE, the E-Box and the CAAT-box were also found in the mouse gene. A region of 21 bp with 100% identity with the mouse major transcription start site was found. It can be assumed that this is also the transcription start site in the human gene. The ATC start site is located 138 bp upstream from the start point of the published cDNA sequence of human *ARNT* (gene bank accession M69238). The human and mouse 5'-flanking region nucleotide sequences show a homology of 67%. The homology of both cDNAs was determined as 89% [Li et al. 1994]. Both the murine and human *AHR* gene 5'-flanking regions are GC rich and do not comprise a TATA-Box or a CAAT-Box [Takahashi et al. 1994; Schmidt et al. 1993].

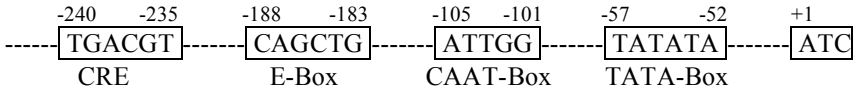


Fig. 3 *ARNT* 5'-flanking region: possible regulatory elements

Acknowledgements

We thank Dr. O. Hankinson, Universitiy of California, Los Angeles, USA for kindly providing a human *ARNT* cDNA clone.

References

Butler, M.A., Lang, N.P., Young, J.F., Caporaso, N.E., Vineis, P., Hayes, R.B., Teitel, C.H., Massengill, J.P., Lawsen, M.F. and Kadlubar, F.F. (1992): Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. Pharmacogenetics 2, 116-127.

Denison, M.S., Fisher, J.M. and Whitlock, J.P. Jr. (1988): The DNA recognition site for the dioxin-Ah receptor complex. Nucleotide sequence and functional analysis. J. Biol. Chem. 263, 17221-17224

Jaeckel, S., Epplen, J.Z., Kauth, M., Miterski, B., Tschentscher, F. and Epplen, C. (1998): Polymerase chain reaction – single strand conformation polymorphism or how to detect reliably and efficiently each sequence variation in many samples and many genes. Electrophoresis 19, 3055-3061

Kawajiri, K., Wanatabe, J., Eguchi, H., Nakachi, K., Kiyohara, C. and Hayashi, S. (1995): Polymorphisms of human Ah receptor gene are not involved in lung cancer. Pharmacogenetics 5, 151-158

Li, H., Dong, L. and Whitlock, J.P. Jr. (1994): Transcriptional activation function of the mouse Ah receptor nuclear translocator. J.Biol.Chem. 269, 28098-28105

Rowlands, J.C. and Gustafsson, J.-Å. (1997): Aryl hydrocarbon receptor-mediated signal transduction. Crit.Rev.Toxicol.27, 109-134

Schmidt, J.V., Carver, L. and Bradfield, C.A. (1993): Molecular characterization of the murine Ahr gene. J. Biol. Chem. 268, 22203-22209

Schrenk, D., Brockmeier, D., Mörike, K., Bock, K.W. and Eichelbaum, M. (1998): A distribution study of CYP1A2 phenotypes among smokers and non-smokers in a cohort of healthy Caucasian volunteers. Eur.J.Clin.Pharmacol. 53, 361-367

Takahashi, Y., Itoh, S., Shimojima, T. and Kamataki, T. (1994): Characterization of Ah receptor promoter in human liver cell line, HepG2. Pharmacogenetics 4, 219-222

Wang, F., Gao, Jx., Mimura, J., Kobayashi, A., Sogawa, K. and Fuji-Kuriyama, Y. (1998): Structure and expression of the mouse AhR nuclear translocator (mArnt) gene. J.Biol.Chem. 273, 24867-24873