CHARACTERIZATION OF REGULATORY ELEMENTS IN THE HUMAN AH RECEPTOR PROMOTER

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Introduction

The Aryl hydrocarbon 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[1]. In the absence of a ligand, the AhR is located in the cytoplasm in a complex with a dimer of heat-shock protein 90 and XAP2 (hepatitis B virus X-associated protein 2). After ligand binding, AhR translocates to the nucleus where it heterodimerizes with the bHLH-PAS protein Arnt (Ah receptor nuclear translocator). The AhR-Arnt heterodimer is able to activate specific xenobiotic regulatory elements (XREs) in enhancers of target genes (e. g. several drug-metabolising enzyme genes). In many mammalian and in several nonmammalian vertebrate as well as in invertebrate species the AhR or homologues have been identified. In humans, it has been found in various tissues such as lung, liver, kidney, placenta and tonsils[2, 3, 4, 5, 6].

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 aimed at the identification and characterisation of regions important for constitutive *AhR* gene expression. For this purpose, we constructed a reporter vector comprising 2.7 kb of the *AhR* 5'-flanking region and deleted various regions. The functional relevance of putative regulatory sequences was investigated by gel mobility shift analysis and site-directed mutagenesis.

Methods and Materials

A PCR-fragment spanning 2.7 kb of the human *AhR* gene 5'-flanking region was amplified with the AdvantageÒ-GC Genomic Polymerase Mix (CLONTECH, Heidelberg, Germany). After ligation in a TA vector (PCR II, Invitrogen, Groningen, Netherlands) it was subcloned in a luciferase reporter vector (pGL3-Enhancer, Promega, Heidelberg, Germany). The identity of the insert was proved 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 baseâ system (Promega, Heidelberg, Germany) according to the instructions of the manufacturer. Internal deletions were generated with specific restriction enzymes followed by S1-nuclease treatment and re-ligation. Site-directed mutagenesis was performed with the QuickChange™ XL Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instruction.

HepG2 cells cultured in DMEM including 20 % FCS and 1 % penicillin / streptomycin were co-transfected with reporter gene constructs and the control plasmid pRL-SV40 (Promega, Heidelberg, Germany), expressing the renilla luciferase gene, using the calcium phosphate co-precipitation method as described previously[7]. Reporter gene assays were performed using the Dual luciferase™ system (Promega, Heidelberg, Germany). Cell homogenates were analysed 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 determined by dividing the firefly luciferase activity (reporter gene) by the renilla luciferase activity (control gene).

Preparation of nuclear extracts was performed as described previously[8].

Gel mobility shift assays were performed with double-stranded synthetic oligonucleotides labelled with [g-^{32}P]ATP using T4 polynucleotide kinase (Promega, Heidelberg, Germany). The binding reaction was carried out with a reaction mixture (15 µl) containing 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 4 % glycerol, 0.5 mM DTT as well as poly(dI-dC) and nuclear extract. After pre-incubation for 20 min, 25 fmole of the labelled oligonucleotide were added and the samples were incubated for additional 30 min. DNA-protein complexes were resolved on a 6 % non-denaturing polyacrylamide gel in 0.8x TBE at 4 °C. Bands were visualized by autoradiography.

Results and Discussion

First, we generated 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 compared with the empty reporter vector.

Using the HUSAR program TRANSFAC we identified several putative binding sites for transcription factors in the flanking region[9]. These factors include e. g. AP1, AP2, CREB, c-Myc, Sp1, and CCAAT binding proteins. Reporter gene assays with various deletion mutants led to the identification of a region containing putative binding sites for Sp1, c-myc and CREB. Two constructs with a deletion in this region, named Del II 2 and Del II 10, were generated. They differ in a region of 46 bp (Figure 1). Interestingly, reporter gene activities of these constructs differed significantly (Figure 2), suggesting that the bases lacking additionally in Del II 10 were functionally relevant.

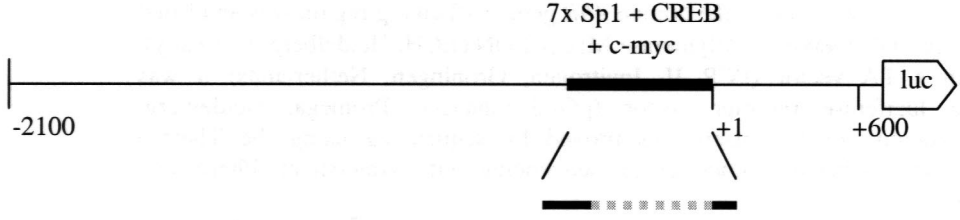

Figure 1. Schematic diagram of the full-length reporter gene construct. Numbers refer to the transcription start site. The dashed region represents lacking bases in Del II 2. Del II 10 is lacking additional bases represented by the bold line.

The functional relevance of the CREB-site was analysed using dibutyryl-cAMP. However, the reporter gene activities were not significantly increased after dibutyryl-cAMP-treatment. This indicates that the CREB-site has no functional relevance.

As Sp1 is described to be involved in the regulation of the murine AhR, it was speculated that it is also involved in the regulation of the human AhR[10].

Therefore, gel mobility shift assays with synthetic oligonucleotides comprising this Sp1 multi-site region were performed. Oligonucleotides with point mutations were used to characterize the binding area for nuclear proteins. In fact, a two-base-substitution in one of the Sp1 sites led to a nearly complete loss of binding of nuclear proteins (Figure 3).

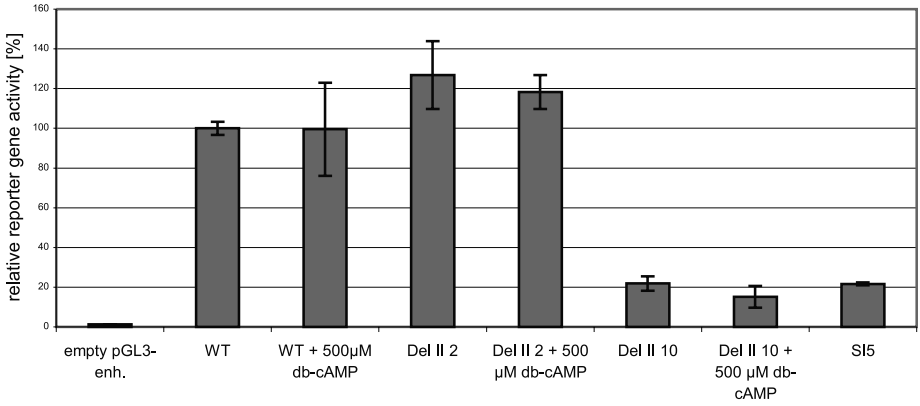

Figure 2. Relative reporter gene activity of wild-type and various deletion constructs with and without treatment with 500 µM dibutyryl-cAMP.

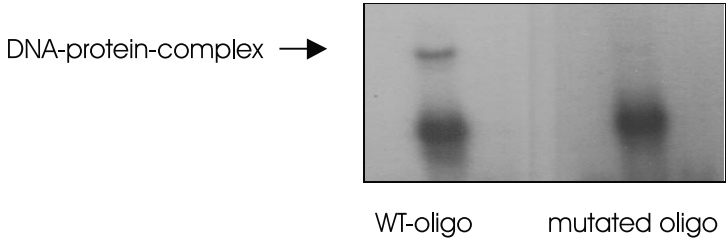

Figure 3. Gel mobility shift assay with nuclear extracts from human HepG2 hepatoma cells using wild-type oligonucleotide and site-directed mutated (in a Sp1-site) oligonucleotide.

Insertion of this mutation into the reporter gene construct (termed SI5) led to a reduction of reporter gene activity of 80% compared to the wild-type construct (Figure 2).

Summarizing, our results indicate that a Sp1-site contributes to AhR regulation. Further experiments including super shift assays, investigation of the influence of Sp1 expression plasmids and of other members of the Sp-family are necessary to reveal the regulatory relevance of the region identified in the present study.

References

1. Schrenk, D., Riebniger, D., Till, M., Vetter, S., Fiedler H. P. (1997): Tryptanthrins: a novel class of agonists of the aryl hydrocarbon receptor. *Biochem. Pharmacol.* 54: 165-171.
2. Butler R. A., Kelley, M. L., Powell, W. H., Hahn, M. E., Van Beneden, R. J. (2001): An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, *Mya arenaria*: evidence that invertebrate AHR homologues lack 2,3,7,8-tetrachlorodibenzo-p-dioxin and b-naphthoflavone binding. *Gene* 278: 223-234.
3. Gu, Y.-Z., Hogenesch, J. B., Bradfield, C. A. (2000): The PAS superfamily: sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* 40: 519-561.

4. Rowlands, J. C., Gustafsson, J.-A. (1997): Aryl hydrocarbon receptor-mediated signal transduction. *Crit. Rev. Toxicol.* 27 (2): 109-134.
5. Bock, K. W. (1994): Aryl hydrocarbon or dioxin receptor: Biologic and toxic responses. *Rev. Physiol. Biochem. Pharmacol.* 125: 1-42.
6. Petrulis, J. R., Hord, N. G., Perdew, G. H. (2000): Subcellular localisation of the aryl hydrocarbon receptor is modulated by the immunophilin homolog hepatitis B virus X-associated protein 2. J. Biol. Chem. 275 (48): 37448-37453.
7. Kauffmann, H.-M., Schrenk, D. (1998): Sequence analysis and functional characterization of the 5'-flanking region of the rat 'multidrug resistance protein 2' (*mrp2*) gene. *Biochem. Biophys. Res. Commun.* 245: 325-331.
8. Kauffmann, H.-M., Vorderstemann, B., Schrenk, D. (2001): Basal expression of the rat, but not of the human, multidrug resistance protein 2 (MRP2) gene is mediated by CBF/NF-Y and Sp1 promoter-binding sites. *Toxicol.* 167: 25-35.
9. Wingender, E., Kel, A. E., Kel, O. V., Karas, H., Heinemeyer, T., Dietze, P., Knuppel, R., Romaschenko, A. G., Kolchanov, N.A. (1997): TRANSFAC, TRRD and COMPEL: towards a federated database system on transcriptional regulation. *Nucleic Acids Res.* 25: 265-268.
10. Fitzgerald, C. T., Nebert, D. W., Puga, A. (1998): Regulation of mouse Ah receptor (AhR) gene basal expression by members of the Sp family of transcriptional factors. *DNA Cell Biol.* 17 (9): 811-822.