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Functional Role of a Constitutively Active Dioxin/Ah Receptor in a Transgenic Mouse Model

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Stockholm 2003

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-469-0

To Charlotte and Linus

Abstract

The dioxin/ Aryl hydrocarbon receptor is a ligand-activated transcription factor that mediates most (if not all) of the toxic effects of the group of highly potent environmental pollutants collectively called dioxins and PCBs, including the highly toxic compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The toxic effects include immune suppression, endocrine disruption, impaired reproduction and carcinogenesis. In addition, the Ah receptor regulates expression of several genes, most of which are xenobiotic metabolizing enzymes such as CYP1A1. Although the high toxicity of these compounds has been known for more than three decades, the mechanism(s) of action behind the wide spectrum of effects is yet not known. Moreover, although the Ah receptor was cloned more than ten years ago, a physiological role or ligand has not unambiguously been identified. A transgenic mouse model that expresses a constitutively active Ah receptor (CA-AhR) was therefore developed. The aim of this study was to characterize the functional role of this receptor in these mice.

Homozygous CA-AhR mice were fertile and the CA-AhR was expressed in several tissues such as thymus, spleen, liver, lungs and heart as well as in all parts of the gastrointestinal tract. In CA-AhR mice, unexposed to any exogenous Ah receptor ligand, expression of the Ah receptor target gene CYP1A1 was induced in all of these organs, indicating that the CA-AhR was transcriptionally active in a constitutive manner. In addition, effects observed in most experimental animals after TCDD exposure, such as decreased thymus weight and increased liver weight were also observed in the CA-AhR mice.

Increased mortality was observed from six months of age in the CA-AhR mice. This correlated with the development of cystic stomach tumors that penetrated all layers of the glandular stomach. These tumors consisted of differentiated cells, such as foveolar, parietal and cardio-pyloral type of cells. Immunohistochemical analysis demonstrated expression of CYP1A1 and the proliferation marker PCNA. Intestinal and squamous metaplasia was also observed in the tumors. Some tumors were surrounded by a region of connective and fatty tissue together with lymphatic foci and vessels, reminiscent of hamartomatous lesions observed in humans. Wild-type animals orally treated with TCDD demonstrated an expansion of the zone of proliferating cells normally found in the narrow isthmus-region of the stomach to include the entire parietal-chief cell region. Moreover, the parietal/chief cell region was decreased in the stomach of CA-AhR mice and this was associated with foveolar hyperplasia.

To identify dysregulated genes in the CA-AhR mouse stomachs, RNA from three months old animals was subjected suppressive subtraction analysis. Osteopontin gene expression was found to be down-regulated in the glandular stomach but also in other organs of CA-AhR mice. TCDD exposure of wild-type and mutant mouse hepatoma cells demonstrated a rapid but transient decrease in osteopontin expression that was dependent on functional Ah receptor and Arnt. Moreover, FACS analysis of lymphoid cells revealed that the CD5-expressing peritoneal population of B cells (B1a) was substantially reduced in CA-AhR mice. Taken together, these results demonstrate that expression of the CA-AhR in mice resulted in a number of effects. In particular, the development of well-differentiated stomach tumors indicates a role of the receptor in regulation of gastric epithelial cell homeostasis.

List of Publications

This thesis is based on the following papers and will be referred to in the text by their roman numerals:

I. Andersson P, McGuire J, Rubio C, Petterson S, Hanberg A and Poellinger L. A constitutively active dioxin/ aryl hydrocarbon receptor induces stomach tumors. (2002) Proceedings of the National Academy of Sciences USA, 99:9990-9995.

II. Andersson P, Rubio C, Kato Y, Poellinger L and Hanberg A. The histologic characteristics of stomach tumors in mice expressing a constitutively active dioxin/ aryl hydrocarbon receptor. Manuscript.

III. Andersson P*, Kuznetsov NV*, Gradin K, von Stein P, Dieckmann A, Pettersson S, Hanberg A and Poellinger L. The dioxin/ aryl hydrocarbon receptor mediates down-regulation of osteopontin gene expression. Manuscript.

IV. Andersson P, Ridderstad A, McGuire J, Pettersson S, Poellinger L and Hanberg A. A constitutively active Aryl hydrocarbon receptor causes loss of peritoneal B1 cells. (2003) Biochemical and Biophysical Research Communications, 302: 336-341, *in press*.

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CONTENTS

LIST OF ABBREVIATIONS

3-MC	3-Methylcholantrene
Ah	Aryl hydrocarbon
AhR	Ah receptor
AhRR	Ah Receptor Repressor
Arnt	Ah Receptor Nuclear Translocator
bHLH	Basic Helix-Loop-Helix
CA-AhR	Constitutively Active Ah Receptor
CD	Cluster of Differentiation
DAB	Diaminobenzidine
ES	Embryonic Stem Cell
FACS	Flourescence Activated Cell Sorter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
HIF-1α	Hypoxia Inducible Factor α
HRP	Horse Radish Peroxidase
Hsp90	Heat Shock Protein 90
IRES	Internal Ribosomal Entry Site
LBD	Ligand Binding Domain
MHC	Major Histocompatibility Complex
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NMO1	NAD[P]H:menadione oxidoreductase
OPN	Osteopontin
PAS	Per-Arnt-Sim
PCB	Polychlorinated Biphenyl
PCDD	Polychlorinated Dibenzo-*p*-dioxin
PCDF	Polychlorinated Dibenzofuran
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription PCR
TCDD	2,3,7,8,-tetrachlorodibenzo-*p*-dioxin
TEF	Toxic Equivalence Factor

Introduction

The dioxin/ Ah receptor

Identification in mouse, human and rat

The dioxin/Ah receptor is a ligand-activated transcription factor that belongs the bHLH/PAS family of transcription factors (see below). The presence of a loci with differing sensitivity for aromatic hydrocarbons was proposed in 1972, based on the observations that polyaromatic hydrocarbons differently induced the enzymatic activity called Aryl hydrocarbon hydroxylase (AHH) in two different inbred strains of mice (the C57Bl/6 and DBA/2) (Nebert et al., 1972). These putative Ah receptor loci were termed $AhR^{b/b}$ (responsive) and $AhR^{d/d}$ (non-responsive) after these mouse strains. By using the more potent inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), it was shown that mice carrying the non-responsive $AhR^{d/d}$ loci actually were responsive, albeit at a ten-fold lower degree compared to the responsive $AhR^{b/b}$ strains (Poland and Glover, 1975). The decreased sensitivity of the $AhR^{d/d}$ allele was later demonstrated to be caused by reduced ligand affinity caused by a mutation in the ligand binding domain (Ema et al., 1994). By the use of radiolabelled ligand, a dioxin-binding protein was identified and was called the Ah receptor (Poland et al., 1976). Since then, two additional alleles of differing size representing responsive Ah receptor forms have been identified in different mouse strains (Poland and Glover, 1990). The cDNA for the mouse Ah receptor was cloned in 1992 by two independent groups (Burbach et al., 1992; Ema et al., 1992). This was quickly followed by cloning of the human (Dolwick et al., 1993) and rat (Carver et al., 1994) Ah receptor. In humans, the Ah receptor is located on chromosome 7p15 (Micka et al., 1997) and in the mouse on chromosome 12 (Poland et al., 1987; Schmidt et al., 1993). In addition, a protein called Ah receptor repressor (AhRR) was recently cloned and shows high homology to the mammalian Ah receptor but does not bind ligand and actually functions as a repressor of Ah receptor function (Mimura et al., 1999).

Ah receptors in other species

Other vertebrate species in which the Ah receptor has been identified (reviewed in (Hahn, 2002) include the beluga whale (Jensen and Hahn, 2001), seals (Kim and Hahn, 2002; Kim et al., 2002) and chicken (Walker et al., 2000). Moreover, bony fish such as the killifish *Fundulus heteroclitus* (Karchner et al., 1999) and the zebra fish *Danio rerio* (Andreasen et al., 2002; Tanguay et al., 1999) as well as cartilaginous fish (Hahn et al., 1997) express two different Ah receptor forms, AhR1 and AhR2. These two Ah receptor forms share some characteristics with the mammalian receptors, such as interaction with partner proteins and transactivation of genes. However, in some species, these receptors may exhibit poor ligand-binding capacity (Hahn, 2002). In addition, a protein showing high sequence similarity to mammalian AhRR has recently been identified in fish (Karchner et al., 2002).

Moreover, proteins showing homology with the mammalian Ah receptors have been cloned from invertebrate species such as the nematode *Caenorhabditis elegans* (Powell-Coffman et

al., 1998), the fruit fly *Drosophila melanogaster* (Duncan et al., 1998) and a soft shelled clam *Mya arenaria* (Butler et al., 2001). The presence of the Ah receptor in three invertebrate phyla (nematodes, arthropods, molluscs) indicate that the AhR should have been present in a common ancestor living up to 970 million years ago (Hahn, 2002). The invertebrate Ah receptors share many structural properties with the vertebrate receptors, but differ by showing poor binding of prototypical ligands (Hahn, 2002).

Fylogenetic analysis of the available Ah receptor sequences indicate that the receptors can be divided into four different "clades": **1.** mammalian AhR and fish AhR1; **2.** fish AhR2; **3.** mammalian and fish AhRR and **4.** invertebrate AhRs (Hahn, 2002). Together with data from studies on ligand-binding and transcriptional activity, a model for the evolution of the Ah receptor is outlined by Hahn (Hahn, 2002): An ancestral AhR (exemplified by the invertebrate AhR of today) was a transcriptional activator that was unable to bind prototypical AhR-ligands (such as dioxins). This gene was duplicated early in vertebrate history and one of these receptors lost ligand-binding and transactivation capacity, acquiring the repressor functions seen in the AhRR, while the other receptor evolved the ability to bind prototypical AhR ligands. An additional gene duplication event occurred, resulting in the additional AhR found in fish.

Expression pattern of the Ah receptor

During mouse embryonic development, the Ah receptor shows a specific expression pattern (Table 1). In several developing organs, Ah receptor protein is detected in the nucleus, suggesting that the receptor is transcriptionally active. Moreover, the expression levels in a certain organ (e.g. the liver) vary between developmental stages (Abbott et al., 1995). This indicates that the Ah receptor may play an important role during embryonic development. In the adult mouse, the Ah receptor is expressed in most organs, including the entire gastrointestinal tract (Andersson et al., 2002; Carver et al., 1994).

Table 1. Expression of the Ah receptor in the developing mouse (Abbott et al., 1995; Jain et al., 1998)**.**

Gestational day	***Expression of Ah receptor***
9.5-10	Neuroepithelium of developing brain, visceral arches, heart
13.5	Primitive pituitary, palatal shelf, nasal septal cartilage, dorsal surface of the tongue, lens of the eye, developing thymus, lung parenchyma, liver, kidney, urogenital sinus, tip of tubercle and epithelia of the developing gut
15-15.5	Nasal septum, surface of the tongue, pituitary, hippocampus, inner layer of retina, thymus, lung mesenchyme, ureteric buds of the kidney, liver, sites of bone formation (e.g. ribs, vertebrae, facial bone), esophagus and the luminal epithelium of the stomach
16	Liver, epidermis, developing bone, regional expression in differentiating kidney and adrenal gland

Ah receptor forms with different sensitivity for TCDD

In addition to the different mouse alleles mentioned above, several different Ah receptor forms that show dramatically different sensitivity to TCDD exist in the rat. The rat strain Han/Wistar (Kuopio) is highly resistant to TCDD exposure compared to most other commonly used rat strains. This resistance is caused by a point mutation resulting in a truncation of the C-terminal transactivation domain (Pohjanvirta et al., 1998). In addition, the difference in sensitivity for the lethal effects of TCDD differ by more than 1000-fold between the most sensitive species, the guinea pig and the least sensitive, the hamster (Poland and Knutson, 1982). Although the Ah receptor has been cloned in these two species (Korkalainen et al., 2000; Korkalainen et al., 2001), the mechanism underlying these differences remain to be elucidated. Although some polymorphisms in the human Ah receptor gene have been identified, a functional role for these mutations has not yet been shown (Harper et al., 2002).

Physiological role for the Ah receptor? – Observations in Ah receptor knockout mice

Three independent research groups have created Ah receptor-deficient mice, with varying phenotypes. In addition to deficient induction of Ah receptor target genes, the first model presented by the Gonzales laboratory demonstrated neonatal mortality of the homozygous pups (up to 40-50% within 1 to 4 days after birth); decreased body weight during first weeks of life; decreased liver weight associated with fibrosis in the portal tract and substantially reduced cell number in the spleen (Fernandez-Salguero et al., 1995). A more extensive analysis of older animals of this AhR-/- variant showed gross enlargement of the heart associated with inflammation and fibrosis as well as hypertrophic uteri that displayed thromboses and mineralization of the serosal vessels (Fernandez-Salguero et al., 1997). In mice older than 9 months focal hyperplasia that progressed to large placques or polyps were commonly observed in the pyloric region of the stomach. In the most severe case, this polypoid lesion extended into the submucosa and was associated with a lymphoid aggregate. Moreover, rectal prolapse was observed in approximately half of the animals and periportal fibrosis was seen in many livers. The zones containing B and T cells in the spleen (the white pulp) was significantly decreased and around half of the AhR-/- mice developed progressive, focal lesions in the dorsal skin that started with alopecia and progressed to ulcers (Fernandez-Salguero et al., 1997).

Analysis of an independently developed Ah receptor knockout mouse showed that some of the effects observed in the report by Fernandez-Salguero et al. (1995) were reproduced, such as decreased body weight during the first weeks of life, decreased liver weights, liver pathology and resistance to target gene induction by TCDD (Schmidt et al., 1996). However, these mice differed in several aspects from the first presented knockout: no neonatal mortality could be observed; there was no decrease in splenic lymphocytes; no lymphocyte infiltration of major organs was seen**,** and these mice exhibited additional pathological lesions in the liver. These included a transient microvesicular fatty change associated with persistence of extramedullary hematopoesis (Schmidt et al., 1996). Possible causes underlying these differences, such as different targeting strategies have been discussed (Lahvis and Bradfield, 1998).

An extended analysis of the AhR-/- mice from the Bradfield laboratory demonstrated several examples of fetal vascular structures remaining in adult animals. The most prominent effect is a shunt in the liver, an open ductus venosus that results in substantially decreased blood flow in the liver sinusoids, which in turn demonstrated a more anastomosing pattern in AhR-/- livers comared to wild-type (Lahvis et al., 2000). Other vascular defects that were reminiscent of fetal structures were the presence of hyaloid arteries in the eye (that supplies the fetal lens with oxygen); exaggerated vessels at the border between the cornea and conjunctiva in the eye and altered vascular pattern in the kidney (Lahvis et al., 2000).

In addition, a third line of Ah receptor deficient mice has been presented. These mice also show impaired growth rate during early life and are resistant to teratogenic responses such as cleft palate and hydronephrosis, commonly observed after maternal exposure to relatively high doses of TCDD (Mimura et al., 1997). The AhR-/- mice developed by Fernandez-Salguero et al. are also resistant to the teratogenic effects of TCDD (Peters et al., 1999). In addition, these mice are resistant to very high doses of TCDD (up to 2000 µg/kg body weight) (Fernandez-Salguero et al., 1996). The mice developed by Mimura et al. are resistant to the carcinogenic effects in skin caused by benzo[a]pyrene (Shimizu et al., 2000), most likely due to the absent induction of the bioactivating CYP1A1 enzyme that most likely is needed for adduct-forming reactive metabolites to form. Thus, these are proof of principle experiments showing the dependency of a functional Ah receptor for toxicity of dioxins and PAHs.

In addition to the effects observed in the liver and vasculature of the Ah receptor deficient mice described above, female AhR-/- mice (originating from the Gonzales laboratory) demonstrate considerably impaired reproductive success, exemplified by the fact that less than half of the AhR-/- dams manage to raise pups to weaning, decreased fraction of live pups and implantations as well as increased mortality of the dams during lactation (Abbott et al., 1999). A partial explanation to the reduced reproductive success in the Ah-/- dams could possibly come from two independent studies on ovarian development in Ah receptor deficient mice (originating from the Bradfield laboratory). Increased number of primordial follicles are seen in 2-3 or 4 days old AhR-/- females (Benedict et al., 2000; Robles et al., 2000). At 53 days of age, a decreased number of antral follicles was observed by one group (Benedict et al., 2000), and a resistance of germ cells to undergo apoptosis under serum-free culture conditions was observed by the other group (Robles et al., 2000). These results indicate that the decreased number of antral follicles observed in the AhR-/- mice may be insufficient to support the hormone synthesis needed during pregnancy and lactation. It is also hypothesized that the Ah receptor may play a central role in the process where naked germ cells are covered by somatic cells during the formation of the primordial follicle (Benedict et al., 2000). Notably, a distinct increase in expression of the pro-apoptotic protein Bax with subsequent Bax-dependnt increase in apoptosis was observed in oocytes incubated with 9,10-dimethylbenz[a]anthracene (DMBA) but not with TCDD (Matikainen et al., 2001). Two binding sites for the AhR/Arnt complex (XREs) have been identified in the Bax promoter, and it has been shown that substitution of the existing guanine or cytosine to an adenine three bases from the core XRE renders the Bax-promoter inducible by TCDD, elegantly demonstrating ligand dependent discrimination between response elements (Matikainen et al., 2001).

The bHLH-PAS protein family

Transcription factors can be classified according to the structure of their DNA-binding domain. One such domain is the basic helix-loop-helix motif (bHLH), which is found in several transcription factors that regulate important biological processes such as proliferation (e.g. Myc/Max), myogenesis (e.g. MyoD, myogenin) and regulation of immunoglobulin genes (e.g. the E2A proteins E12 and E47) (Littlewood and Evan, 1995). The DNA binding properties of this motif is dependent on the basic region. The HLH region mediates dimerization with partner proteins which is also required for efficient DNA binding.

A subgroup of the bHLH factors harbors a second structural motif, the PAS domain, which is subdivided in two hydrophobic repeats, PAS-A and PAS-B (Fig. 1). PAS is an acronym of the first three proteins identified containing this motif: the *Drosophila* protein Period (Per), human Arnt and *Drosophila* Single-minded (Sim) (Crews, 1998). The bHLH/PAS family is a growing group of transcription factors that seem to have evolved to respond to different environmental cues (Gu et al., 2000). Besides the ligand-activated Ah receptor, this family contain proteins that respond to low levels of oxygen (Hypoxia Inducible Factors, HIFs), participates in the regulation of circadian rhythms, such as the proteins Cycle and Per, as well as acting as transcriptional coactivators such as SRC-1, reviewed by (Gu et al., 2000).

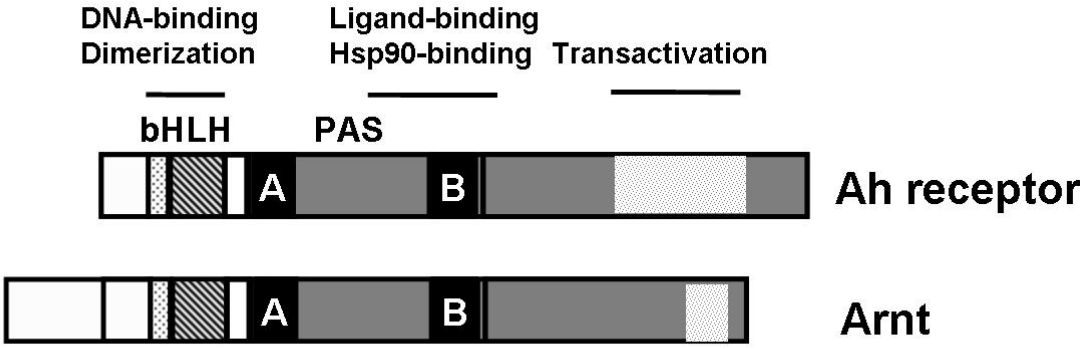

Figure 1. Schematic representation of the Ah receptor and Arnt proteins, indicating functional domains.

Arnt

The first evidence of an accessory factor to the putative Ah receptor came from a screen for mutants of the mouse hepatoma cell line Hepa1c1c7 that were resistant to the induction of AHH activity by aromatic hydrocarbons (Legraverend et al., 1982). One of the resistant clones (C4) was believed to be deficient in ligand-induced translocation of the Ah receptor to the cell nucleus. In subsequent complementation studies where the AHH activity was rescued by transfection of human genomic DNA fragments, the cDNA of the Ah receptor nuclear translocator was cloned (Hoffman et al., 1991). However, the original idea that Arnt participated in nuclear translocation of the Ah receptor has been modified by

immunohistochemical studies showing that nuclear translocation of the Ah receptor in C4 cells is not impaired (Hord and Perdew, 1994; Pollenz et al., 1994). The expression pattern of Arnt seems to be quite ubiquitous, both in adult (Carver et al., 1994) and developing (Jain et al., 1998) rodents. The Arnt gene is located on human chromosome 1q21 and on mouse chromosome 3 (Johnson et al., 1993).

In analogy to the shared dimerization partner for many nuclear receptors, the RXR protein (Mangelsdorf and Evans, 1995), the bHLH/PAS protein Arnt (or its orthologs) functions as an obligate partner for many of the other factors in this family such as the Ah receptor (Probst et al., 1993; Whitelaw et al., 1993a), Hypoxia inducible factors, the mouse proteins Sim, and CLOCK as well as the Per protein (Crews, 1998). The central role for Arnt was demonstrated when mice deficient in Arnt were found to die at gestational day 10.5 due to defective formation of blood vessels (Maltepe et al., 1997). This finding was interpreted as a failure to support signalling of Hypoxia Inducible Factor 1α (HIF-1α) during organogenesis, and this notion was substantiated by the failure of HIF-1α embryos to form a vasculature (Ryan et al., 1998).

Two additional proteins with high homology to Arnt have been cloned. Expression of Arnt2 seems to be restricted to brain and kidney in adult mice and it binds to and it can form a functional transcription unit with the Ah receptor (Drutel et al., 1996; Hirose et al., 1996). Arnt3 (also called MOP3 "Member Of PAS" or BMAL1 "Brain and Muscle Arnt-Like protein 1) can dimerize with HIF-1α and circadian rhythm factors such as clock (Hogenesch et al., 1998; Ikeda and Nomura, 1997), and has been shown to be an essential component of the mammalian circadian pacemaker (Bunger et al., 2000).

Ah receptor ligands

The Ah receptor binds a large number of ligands of varying origin, structure and physico-chemical properties (Denison et al., 2002). Many ligands come from anthropogenic sources, others are derived from the diet and some are endogenously formed within the body. Compounds also exist, which can modulate the transcriptional activity of the Ah receptor, without yet having been shown to be true ligands (Denison et al., 2002). Despite this, none of the identified ligands have yet proven to be a true physiological ligand relevant for Ah receptor biology in vivo, and the physiological function of the receptor remains elusive.

Persistent organohalogen pollutants

The best characterized Ah receptor ligands are members of the classes of environmental pollutants that collectively are called dioxins and PCBs (Safe, 1990). These compounds are aromatic hydrocarbons that are halogenated at various positions, resulting in different congeners that show varying toxicological potency (Fig 2). The dioxins consist of 210 different polychlorinated dibenzofurans (PCDF) and dibenzo-*p*-dioxins (PCDDs), including the most toxic congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The polychlorinated biphenyls (PCBs) constitute 209 different congeners. Dioxins have never been commercially produced but have unintentionally been formed during combustion (e.g. waste incineration), chlorine bleaching of paper as well as in the production of other chemicals such as

chlorophenols and phenoxy acetic acids, exemplified by the defoliant Agent Orange used in the Vietnam War. In contrast, the PCBs have been used extensively in a wide spectrum of applications, such as insulator material in electrical capacitors and transformers, but also in building materials. However, due to the identification in the 60's of the persistency and bioaccumulation and in the 70's the highly toxic properties of these chemicals, the production and use of PCBs was banned in most Western countries during the 80's. Precautionary measures have been taken to reduce the formation and excharge of dioxins.

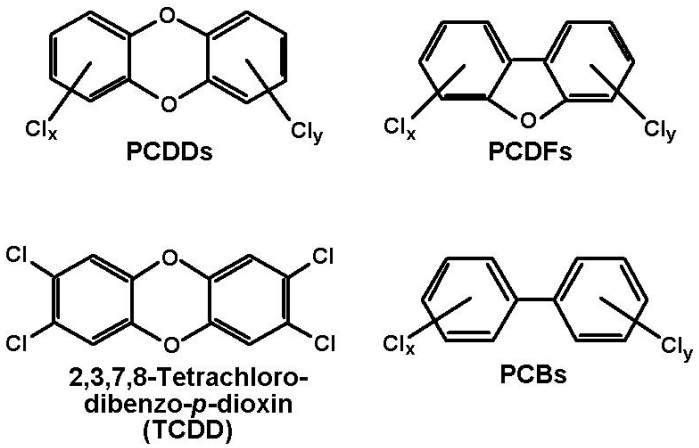

Figure 2. Chemical structure of polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofurans (PCDF), TCDD and polychlorinated biphenyls (PCB).

Dioxins and PCBs that are highly chlorinated in the lateral positions accumulate in the food chains since they are very lipophilic and resistant to both biological and chemical degradation. Consequently, food is the major route of exposure for humans, accounting for up to 90% of the total exposure (Liem et al., 2000). In light of the numerous sites contaminated by dioxins and PCBs, the recently reported identification of an anaerobic bacterium that is capable of dehalogenating chlorinated dioxins (Bunge et al., 2003) warrants hope for easier clean-up procedures.

Many of the large number of chemicals that demonstrate dioxin-like structures cause a similar spectrum of toxic effects, although at considerably different dose levels. They are often found in the environment as constituents of complex mixtures which is also the way that humans and wildlife are exposed. In order to encompass all these compounds in risk assessment, the so-called TEF-concept was developed (Ahlborg et al., 1994; van den Berg et al., 1988; Van den Berg et al., 1998). This concept uses the toxicity of TCDD as a reference, which is assigned a toxic equivalence factor (TEF) of one. The potency of chemicals that falls within the applied criteria (i.e.structural relationship to dioxins; Ah receptor binding; Ah receptor-mediated toxic and biochemical responses and is persistent and accumulates in food chains) can then be compared to that of TCDD and be assigned a TEF value (e.g. 0.01 for a compound that is 1/100 as potent as TCDD). The TEF value is multiplied with the concentration of the corresponding chemical in a mixture, and these values can then be summarized in order to quantify the toxic equivalence (TEQ) of the complex sample expressed in the "TCDD-unit" TEQ.

Polyaromatic hydrocarbons (PAH)

Polyaromatic hydrocarbons, such as 3-methylcholantrene, benz[a]pyrene and dibenz[a,h]anthracene bind and activate the Ah receptor (Okey et al., 1984; Vos et al., 1982). However, these compounds are metabolized by enzymes (such as CYP1A1) that are induced by the activated Ah receptor, thereby stimulating their own degradation. Moreover, this CYP-mediated metabolism can also cause formation of highly reactive diol-epoxides that easily form adducts with proteins and DNA, thereby initiating chemical carcinogenesis.

Ligands derived from the diet

The majority of identified Ah receptor ligands of natural origin are derived from the diet, especially from plants. Many compounds that are weak receptor ligands (e.g. flavonoids, carotinoids, phenolic compounds) can still activate Ah receptor signalling pathways, and a number of substances that act as ligands/agonists or antagonists that are present in different fruits, vegetables, herbs and teas have been reported (Denison et al., 2002). Interestingly, conversion in the gastrointestinal tract of dietary indoles, including indole-3-carbinol (I3C) and tryptophan can generate potent Ah receptor ligands (Bjeldanes et al., 1991; Perdew and Babbs, 1991). One of the condensation products of I3C, Indolo-[3,2-b]-carbazole (ICZ) is a highly potent ligand and activator of the transcriptional activity of the Ah receptor (Kleman et al., 1994). In addition, analysis of activation of the human, but not mouse Ah receptor by the proton-pump inhibitor omeprazole led to the conclusion that omeprazole is a precursor of an actual Ah receptor-binding ligand (Dzeletovic et al., 1997).

Endogenously formed ligands

Recently, 20 µg of a putatively endogenous Ah receptor ligand was isolated from 35 kg of porcine lung. This compound, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) activates the Ah receptor with a potency five times higher than β-naphtoflavone and is able to compete with TCDD for binding to human, mouse and fish Ah receptors (Song et al., 2002). This finding is very intriguing, considering the distinct expression of Ah receptor in the lungs of both the developing (Abbott et al., 1995; Jain et al., 1998) and adult (Andersson et al., 2002) mouse. Ligands that demonstrate high-affinity binding to the Ah receptor are formed when tryptophan is exposed to UV-light (Rannug et al., 1987; Rannug et al., 1995). Interestingly, these ligands (6,12-diformylindolo[3,2-b]carbazole (dFICZ) and 6-formylindolo[3,2-b]carbazole (FICZ) show high structural similarity with the potent ligand (ICZ), formed in the gastrointestinal tract. Moreover, indirubin and indigo have been identified in human urine and can also activate the Ah receptor (Adachi et al., 2001).

Ah receptor activation

Activation of the Ah receptor is a multistep process, which has been extensively studied in the context of activation of the CYP1A1 promoter, reviewed in (Whitlock, 1999). In the absence of ligand, the Ah receptor is located in the cytoplasm in a complex (Fig. 3) with a dimer of the chaperone protein Hsp90 (Denis et al., 1988; Perdew, 1988) and the immunophilin-related protein XAP2 (HBV X-associated protein 2) also known as ARA9 (AhR associated protein 9)

and AIP (AhR interacting protein) (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al., 1998). This protein has been shown to stabilize and keep the receptor in the cytoplasm, protecting it from ubiquitination and subsequent degradation by the 26S proteasome (Kazlauskas et al., 2000; Petrulis et al., 2000), possibly by inhibiting association with importin, a protein responsible for nuclear import (Petrulis et al., 2002). Expression levels of XAP2 vary considerably between tissues, and it has been proposed that alternate XAP2 levels could contribute to the observed variability in organ sensitivity for dioxin toxicity (Petrulis and Perdew, 2002). In addition, the cochaperone p23 has been shown to associate with the Ah receptor bound Hsp90 dimer (Kazlauskas et al., 1999).

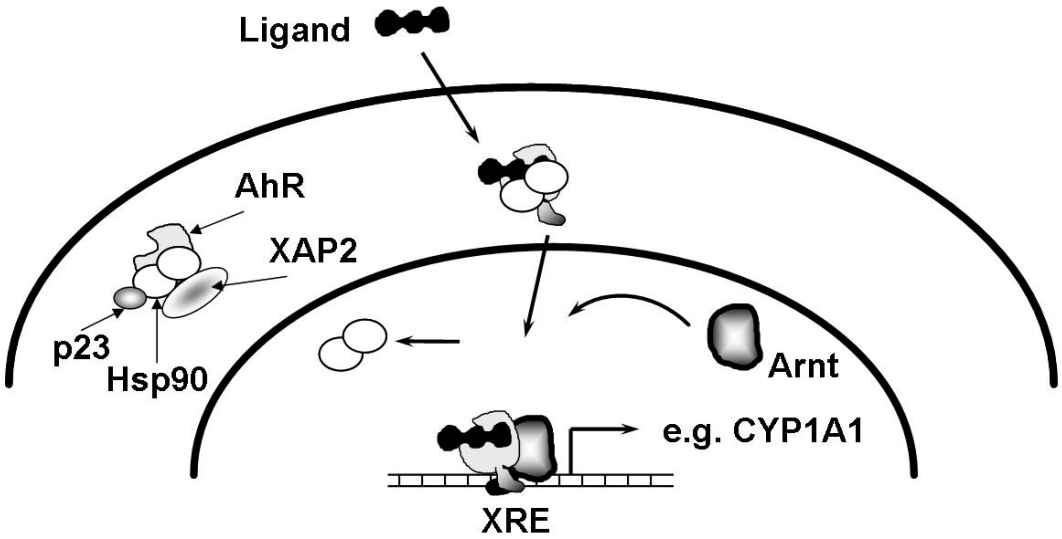

Figure 3. Schematic illustration of Ah receptor activation.

Binding of Hsp90 to the Ah receptor renders it incapable of binding DNA, but seem to be a prerequisite for high-affinity binding of ligand (Antonsson et al., 1995; Pongratz et al., 1992). Hsp90 interacts with the Ah receptor in both the bHLH and PAS B regions (Antonsson et al., 1995; Whitelaw et al., 1993b). Moreover, the core ligand binding domain has been mapped to residues 230-421 overlapping with the Hsp90-interacting region (Coumailleau et al., 1995; Whitelaw et al., 1993b) (Fig. 1).

Ah receptor ligands are lipophilic and diffuse passively over the cell membrane into the cytosol where they bind to the Ah receptor. This binding causes a poorly understood transformation of the receptor, in which the nuclear localization signal (NLS) located within the bHLH region (Ikuta et al., 1998), possibly becomes exposed (Kazlauskas et al., 2001). The AhR/Hsp90-complex is then translocated into the nucleus where binding of Arnt displaces the Hsp90 (Fig. 3) (McGuire et al., 1994). Both the bHLH and PAS regions of the Ah receptor and Arnt are needed for stable association between these proteins (Lindebro et al., 1995; Reisz-Porszasz et al., 1994; Whitelaw et al., 1993b). The ligand-AhR/Arnt complex is now capable of high-affinity binding by their bHLH-regions to so called xenobiotic (or dioxin) response elements (XRE or DRE) within the regulatory regions of target genes (Whitelaw et al., 1993a). The consensus core sequence of the XRE-sequence is 5'-TNGCGTG-3' (Fujisawa-Sehara et al., 1987). The Ah receptor/Arnt complex interacts with

several different cofactors that modulate the level of transcription. Some of these proteins harbor histone acetyltransferase activity (HAT), such as SRC1, NcoA-1/GRIP-1/TIF-2 and CBP/p300 (Beischlag et al., 2002; Kobayashi et al., 1997). In addition interactions have also been shown with the transcriptional coactivator RIP-140 as well as BRG-1 and Brahma of which the two latter are members of the chromatin remodelling complex SWI/SNF (Kumar et al., 1999; Wang and Hankinson, 2002). Together, these proteins contribute to a more open chromatin structure and/or recruit the basal transcription machinery to the transcriptional start site, thereby initiating transcription of the target genes.

In the presence of TCDD, the majority of Ah receptor protein can be recovered in the nucleus within 30 to 60 minutes after exposure, followed by a rapid decrease in the nuclear compartment resulting in essentially no nuclear Ah receptor 4 to 6 hours after exposure (Pollenz, 2002). The cellular half-life of the Ah receptor is approximately 28 hours in the absence of ligand and 3 hours in the presence of ligand (Ma and Baldwin, 2000). This decline in receptor levels has been observed in cell cultures as well as in intact animals and is an important step in regulation of the duration and magnitude of the transcriptional response (Pollenz, 2002). The Ah receptor is believed to be exported from the nucleus via interaction with the CRM-1 export protein and subsequently degraded by the cytosolic 26S proteasome, reviewed in (Pollenz, 2002). In contrast, the levels of Arnt appear to be unaffected (Pollenz, 2002). Interestingly, two nuclear export signal (NES), has been identified of which one is located in the N-terminal part of the PAS domain of the Ah receptor (Berg and Pongratz, 2001).

In addition to the previously mentioned regulatory steps in Ah receptor activation, the presence of the Ah receptor repressor protein (AhRR) adds another regulatory dimension. This protein was initially discovered as a homologous protein that also was a target gene of the activated Ah receptor (Mimura et al., 1999). The expression of AhRR is regulated by binding of the AhR/Arnt complex to three relatively weak XRE-sequences located in the promoter region of the AhRR gene (Baba et al., 2001). The AhRR shows high sequence homology to the Ah receptor in the bHLH and PAS A regions, but lacks the transactivation domain and ligand binding motif contained within the PAS B region (Mimura et al., 1999). The AhRR dimerizes with Arnt in a constitutive manner, and this complex specifically binds XRE-sequences. These properties led to a proposed negative-feedback model in which the activated Ah receptor induces transcription of the AhRR, which complexed with Arnt functions as a competitor for binding to XRE sequences in target genes (Mimura et al., 1999). Increased expression of AhRR in 3-methylcholantrene exposed mice is rapidly observed in lung and heart but at substantially lower levels in thymus, liver, kidney and intestine (Mimura et al., 1999). Interestingly, constitutive expression is observed in the stomach of untreated mice (Andersson et al., 2002), possibly caused by Ah receptor activation of ligands derived from the rodent diet. The chromosomal locations of AhRR gene are13C2 in mice, 1p11.2 in the rat and 5p15.3 in humans (Baba et al., 2001). The physiological role of the AhRR is poorly understood but a polymorphism has been suggested to be associated by the phenomenon of micropenis in humans (Fujita et al., 2002). In addition, an Arnt-containing protein complex has been identified to bind XRE sequences in human fibroblasts that are resistant to the target gene activating effects of dioxins (Gradin et al., 1999). Intriguingly, the protein associated with Arnt may possibly constitute the AhRR.

Ligand-independent activation

Human keratinocytes demonstrated a substantial increase in Ah receptor target gene expression when they were cultured in suspension (Sadek and Allen-Hoffmann, 1994a). This phenomenon appears to be mediated by the Ah receptor/Arnt complex since mutant hepatoma cells with either decreased Ah receptor levels or non-functional Arnt protein displayed this increase in CYP1A1 (Sadek and Allen-Hoffmann, 1994b). Moreover, observation of constitutively nuclear and transcriptionally active Ah receptor in CYP1A1 deficient mouse hepatoma or CV-1 cells has been interpreted to support the notion of an endogenous Ah receptor ligand that is degraded by CYP1A1 (Chang and Puga, 1998). However, the identity and functional role of this putative ligand remain to be discovered.

A constitutively active Ah receptor – the CA-AhR

Deletion of the ligand binding domain (LBD) of the mouse Ah receptor (residues 230 to 421) results in a protein that does not interact with Hsp90 within the PAS domain and shows constitutive nuclear localization (McGuire et al., 2001). However, this mutant fails to dimerize with Arnt and consequently also failed to bind DNA. The C-terminal part of the LBD (residues 288-421) encompasses the full PAS B region. Deletion of these amino acids gives a mutant that not only fails to interact with Hsp90 in the PAS B region and shows constitutive nuclear localization, but also binds Arnt and exhibits potent transactivation in a constitutive manner in the absence of ligand (McGuire et al., 2001). Conditional expression of the CA-AhR (by the tet-off system) in the human breast cancer cell line MCF-7 results in increased expression of CYP1A1 as well as inhibition of estrogen-stimulated proliferation of these cells (Köhle et al., 2002). Moreover, the transcriptional activity of an estrogen receptor fused to a Gal4 DNA binding domain is inhibited, possibly due to competition of a limited pool of shared co-activators (Köhle et al., 2002).

Ah receptor target genes

The upregulation of a battery of six enzymes involved in biotransformation of xenobiotic and endogenous compounds in response to Ah receptor ligands such as TCDD and 3-MC has been known for a long time and studied in several species (Nebert, 1989). This battery includes the cytochrome P450 enzymes CYP1A1 and 1A2; glutathione transferase Gsta1 (Ya); NAD[P]H:menadione oxidoreductase or DT-diaphorase (Nmo1); UDP glucuronosyltransferase 1A6 (Ugt 1A6); cytosolic aldehyde dehydrogenase 3 (Aldh3a1), reviewed in (Nebert et al., 1990; Nebert et al., 2000; Schrenk, 1998). In addition, a third cytochrome P450 enzyme, namely CYP1B1, has also been identified as a target gene for the Ah receptor (Savas et al., 1994; Shen et al., 1993; Sutter et al., 1994). The three cytochrome P450 enzymes are NADPH-dependent monooxygenases that mediate phase I metabolism, rendering the end-product more reactive for subsequent reactions. The other four enzymes belonging to this group participates in phase II metabolism by conjugating e.g. glutathione or glucuronosyl groups to chemicals to facilitate excretion. Many potent Ah receptor ligands (e.g. PAHs) are metabolized by these enzymes and, not surprisingly, the idea that the Ah receptor may function as a chemical sensor for hazardous chemicals encountered in the environment has, not surprisingly, been proposed (Gu et al., 2000).

Induction of the enzymes listed above cannot explain the plethora of toxic effects that are seen in experimental animals after exposure to e.g. TCDD (see below). Ah receptor ligand-induced alteration of gene expression is seen a number of genes that do not code for metabolizing enzymes. Some of these genes have proven to be "true" Ah receptor target genes, i.e. being transcriptionally regulated by the AhR/Arnt complex bound to XRE-sequences in the gene regulatory region. Altered transcription of other genes is affected by a non-XRE mediated mechanism. However, the finding that exposure to benzo[a]pyrene, but not TCDD, results in XRE-mediated induction of the proapopotic gene Bax in oocytes (Matikainen et al., 2001), demonstrates that sequences outside the core XRE may direct Ah receptor mediated transcription of genes in a ligand-specific manner, adding an additional level of complexity when evaluating potential Ah receptor target genes.

Several different methodological approaches have been used to discover new Ah receptor target genes, such as microarrays (Frueh et al., 2001; Martinez et al., 2002; Puga et al., 2000b; Thomas et al., 2001), differential display (Dong et al., 1997; Kietz and Fischer, 2003; Svensson and Lundberg, 2001), representational diference analysis (RDA) (Ohbayashi et al., 2001; Oikawa et al., 2002) and serial analysis of gene expression (SAGE) (Kurachi et al., 2002). In addition, TCDD-induced differences in protein expression have been analyzed by two-dimensional gel electrophoresis (Ishimura et al., 2002). Examples of more recently discovered genes that are differentially expressed as a result of exposure to Ah receptor ligands are presented in table 2.

Table 2. *Genes recently discovered to be regulated by Ah receptor ligands*

Gene	Up or Down	Function	Refence
Adseverin	Up in thymus	Actin binding	(Svensson and Lundberg, 2001)
Ah receptor repressor	Up in e.g. lung, heart	Ah receptor repressor	(Mimura et al., 1999)
DIF-3	Up in testis	unknown	(Ohbayashi et al., 2001)
IgE-dependent histamine releasing factor (HRF)	Up in mouse embryonic stem cells (ES)	Histamine release	(Oikawa et al., 2002)
N-myristoyltrans-ferase 2 (NMT2)	Up in 5L cells and liver	protein myristoylation	(Kolluri et al., 2001)
$_{P}$27/kip1	Up in 5L cells and thymus	Cell cycle inhibitor	(Kolluri et al., 1999)
γ-catenin	Down in WB-344 cells	Tumor suppressor.	(Dietrich et al., 2003)

Ah receptor-mediated toxicity

The overall toxicity of the prototypical Ah receptor ligands, such as TCDD, has been the subject of several extensive reviews (IARC, 1997; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982) and will only be covered briefly here.

Sensitivity for many of the toxic effects caused by dioxins, PCBs and PAHs was early shown to segregate with the Ah receptor locus, indicating the importance of this receptor in mediating these effects (Poland and Knutson, 1982). Most of what is known about the toxicity of TCDD and related compounds come from studies in experimental animals, but effects are also observed in humans. The most dramatic effect is a lethal wasting syndrome observed at high TCDD doses, extensively reviewed in (Pohjanvirta and Tuomisto, 1994). Lethality is also one of the toxic effects where the largest species and strain differences are seen (Birnbaum and Tuomisto, 2000; Pohjanvirta and Tuomisto, 1994). The liver has been extensively studied where hyperplasia, fatty infiltration and necrosis have been reported in several species and hepatic accumulation of porphyrin (a heme precursor) is also observed (Birnbaum and Tuomisto, 2000). Multiple hormone systems are disturbed by TCDD: levels of thyroid hormones are decreased, and TCDD exposure result in a number of anti-estrogenic effects as well as impaired reproduction (Birnbaum and Tuomisto, 2000). Several different effects are also seen in the Vitamin A homeostasis, including depletion of the hepatic Vitamin A stores (Nilsson and Hakansson, 2002). Cleft palate, hydronephrosis, reduced sperm counts, impaired mating behaviour and multiple malformations of the urogenital tract such as vaginal thread and cleft phallus in females have been observed after prenatal TCDD exposure (Birnbaum and Tuomisto, 2000). In addition, TCDD exposure of rats and mice in utero or during lactation impairs development of the prostate (Lin et al., 2002; Roman and Peterson, 1998; Roman et al., 1998).

TCDD has been analyzed for potential mutagenic effects but is considered to be non-genotoxic (Dragan and Schrenk, 2000). Instead, TCDD seems to act as a tumor promoter, both in skin of hairless mice and in liver (Dragan and Schrenk, 2000; IARC, 1997). Numerous epidemiological studies have been performed on populations that have been exposed to increased or background levels of dioxins and related compounds. There is no cancer type that predominately shows association with this dioxin exposure; it is instead an increased risk for all cancers combined that is the characteristic of dioxin carcinogenicity (Kogevinas, 2000). TCDD is classified by IARC as carcinogenic to humans (group 1) and TCDD is described as "a multi-site carcinogen in experimental animals that has been shown by several lines of evidence to act through a mechanism involving the Ah receptor" (IARC, 1997).

The immune system is a very sensitive target for the adverse effects of dioxins (Kerkvliet, 1995). Atrophy of the thymus is observed in most laboratory animals (Poland and Knutson, 1982). TCDD clearly perturbs normal processes within the immune system, causing suppressed proliferation of B-cells and decreased antibody production (Holsapple et al., 1991). In addition, exposure to TCDD results in decreased resistance against several infectious agents, such as influenza virus (Burleson et al., 1996) and *Thrichinella spiralis* (Luebke et al., 1994).

Relatively recently discovered targets for dioxins are teeth and bone. In rats exposed in utero to TCDD, both dentin formation and tooth eruption are inhibited (Kattainen et al., 2001; Kiukkonen et al., 2002). Exposure of rats shows that TCDD dose-dependently interferes with growth, modeling, and mechanical strength of bones, in this case the tibia (Jamsa et al., 2001). Moreover, cultured bone cells exposed to TCDD (Gierthy et al., 1994; Singh et al., 2000) or 3-methylcholantrene (Naruse et al., 2002) demonstrate impaired differentiation as shown by inhibited expression of markers for mature bone cells such as alkaline posphatase, osteocalcin and osteopontin.

In a relatively early review of the toxicity of TCDD (Poland and Knutson, 1982), epithelial cells were pointed out as being one of the prime targets for dioxins. In fact, one of the most characteristic effects observed in humans exposed to (high) levels of dioxins is chloracne, which is characterized by a hyperplastic and hyperkeratotic response with squamous metaplasia of sebaceous glands in the skin, resulting in severe cystic, acne-like lesions (Poland and Knutson, 1982). Interestingly, TCDD exposure can cause squamous metaplasia in the endocervix of cynomolgus macaques, i.e. a nonhuman primate (Enan et al., 1998; Scott et al., 2001).

A number of different mechanisms have been proposed in order to explain the toxic effects by TCDD and related compounds. Since the Ah receptor is a transcription factor, dysregulation of genes important for e.g. differentiation, migration, proliferation or apoptosis are most likely involved. However, none of the target genes identified to date has unambiguously been shown to mediate the toxic responses observed. Obviously, the receptor can also affect other signal transduction pathways by alternative mechanisms. For example, the Ah receptor can interact with NF-kB signalling (Tian et al., 2002) and with the retinoblastoma protein, Rb (Ge and Elferink, 1998; Puga et al., 2000a). Sharing the same partner factor Arnt, Hif-1α and the Ah receptor signalling pathways have been shown to functionally interfere with one another (Chan et al., 1999; Gradin et al., 1996). Increased level of oxidative stress has also been proposed to contribute to the toxic effects by TCDD (Dalton et al., 2002).

Interestingly, embryonal fibroblasts derived from Ah receptor deficient mice (Elizondo et al., 2000) show increased doubling time, associated with a decrease in mRNA levels for Cdc2 and Plk, proteins that are involved in the cell cycle. Moreover, mutant mouse hepatoma cells with substantially reduced Ah receptor levels (Ma and Whitlock, 1996) also demonstrate increased doubling time, which is normalized when the Ah receptor is expressed by transient transfection. In contrast, exposure of the human lung carcinoma cell line A549 to the Ah receptor ligand β-naphtoflavone results in mildly increased proliferation that also was observed in stably transfected cells overexpressing the receptor (Shimba et al., 2002). This effect is associated with increased mRNA expression of the proliferation marker PCNA (Shimba et al., 2002). Thus, even though no overt signs of impaired cell cycle control have been reported from any of the Ah receptor deficient mice, it is possible that, under certain conditions, the receptor may play an important role in regulation of the cell cycle.

Toxic effects by Ah receptor ligands in the gastrointestinal tract

The gastrointestinal tract of rodents differs from the human counterpart by the thin-walled proximal part of the stomach, the forestomach (Fig. 4). This part is constituted by squamous epithelium and is separated from the glandular part of the stomach by a narrow structure called the limiting ridge (Fig. 4C). Besides having these structures and a caecum of significantly larger size, rodents have a gastrointestinal tract including esophagus, stomach, duodenum, jejunum, ileum and a colon that is anatomically comparable to humans (Fig. 4A). The overall organization of the gastrointestinal tract is the same from the esophagus to the colon and rectum, as exemplified by a schematic illustration of the zymogenic part of the glandular mucosa (Fig. 4B). The lumen is lined by epithelial cells that are squamous in the esophagus, organised in villiform structures in the intestine and organized in a mucous-, enzyme-, and acid-secreting mucosa in the stomach. These epithelial cells rest on a thin muscle layer, the muscularis mucosa, which in turn is overlaying the submucosa, which mostly is constituted by connective tissue (Fig 4B). Underneath the submucosa are several muscle layers located; the muscularis propria, which in turn is demarcated from the peritoneal cavity by the thin subserosa.

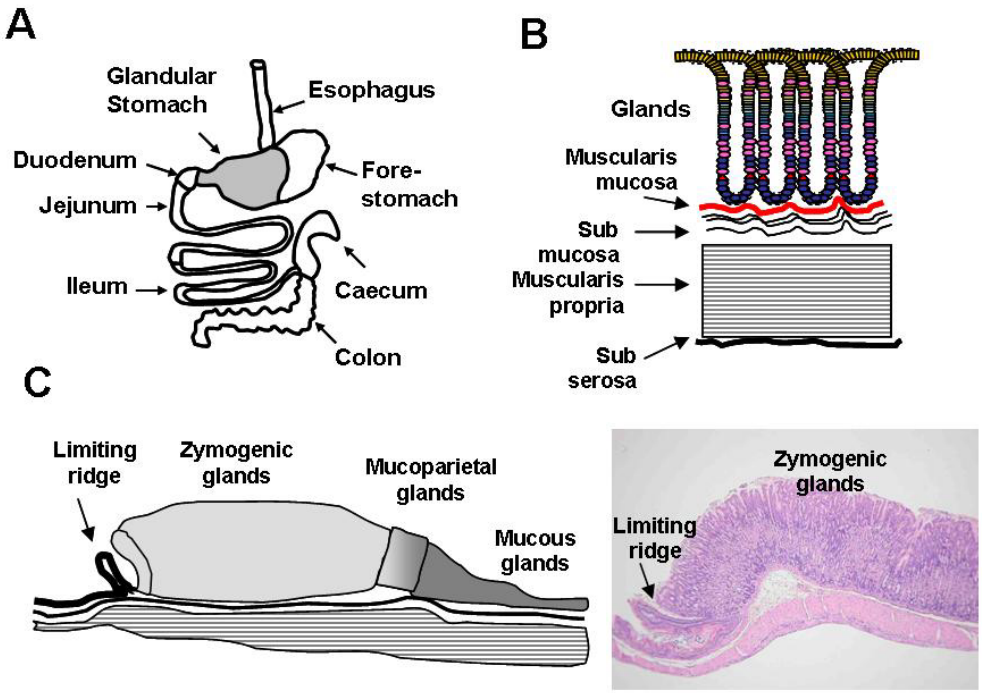

Figure 4. A. The rodent gastrointestinal tract. ***B.*** Schematic illustration of the different tissue layers in the gastrointestinal tract. ***C.*** Cartoon and real section of a mouse stomach showing some important features.

There is an extensive turnover of cells in the gastrointestinal tract. New cells are born in designated areas, which in the case of the zymogenic gastric mucosa, is called the isthmus region (Fig. 5). During their migration these cells differentiate into more specialized cells,

such as mucous-secreting foveolar pit cells, H^+-secreting parietal cells and zymogenic/chief cells that secrete pro-enzymes such as the pepsin-precursor pepsinogen II. The foveolar pit cells migrate towards the lumen and die by apoptosis and /or necrosis and are sloughed off. It is estimated that they complete that process in three days (Lorenz and Gordon, 1993). The other, more specialized cell types remain longer in the mucosa, exemplified buy the 194 days average turnover time for chief cells. It seems like parietal cells can be formed from the stem isthmus stem cell but also from the pre-pit precursors and the pre-neck precursors (Fig. 5).

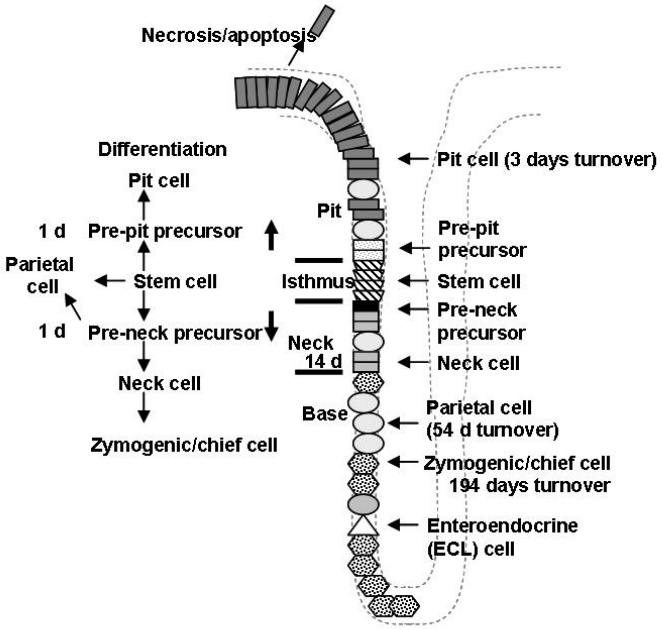

Figure 5. A zymogenic gland of the stomach mucosa, showing the differentiation pathways on the left. Adapted after (Lorenz and Gordon, 1993).

Considering that the gastrointestinal tract is an organ system, which at certain organizational levels (e.g the isthmus), exhibits a high rate of proliferation with subsequent rapid cell differentiation, it should be prone to be affected by multifunctional toxic chemicals such as dioxins and PCBs. In fact, in the review by Poland and Knutson (1982), several examples of hyperplastic lesions in the stomach of monkeys and cows (!) are described. Today, the stomach is rarely mentioned as a target organ for Ah receptor mediated toxicity. However, numerous examples exist where lesions in the stomach have been observed. Exposure to commercial mixtures of PCBs (Aroclors) resulted in stomach lesions in rhesus monkeys (Allen et al., 1974; Allen and Norback, 1973; Becker et al., 1979; McConnell et al., 1979) and in rats (Morgan et al., 1981; Ward, 1985). This is also seen in rhesus monkeys (Becker and McNulty, 1984; McNulty et al., 1980; Silverman et al., 1979) and marmoset monkeys (van den Berg et al., 1988) exposed to the dioxin-like 3,4,3'4'-TCB (PCB77). Exposure of rhesus monkeys (Lambrecht et al., 1978) and rats (Gupta et al., 1983) to commercial mixtures of polybrominated biphenyls also resulted in similar lesions. Finally, exposure to TCDD-like dibenzofurans elicited stomach lesions in rhesus monkeys (Brewster et al., 1988; McNulty et al., 1981) and in hairless mice (Hebert et al., 1990).

A similar pattern can be found in all these studies. There is a marked hyperplasia of the surface mucous cells and a remarkable decrease in the number of parietal and chief cells. In most studies, cystic dilatations are seen at the base of the gastric glands and in the majority of the reports, glandular structures penetrate into the submucosa where they often are associated with foci of lymphatic cells. Intestinal metaplasia, as assessed with Alcian blue-staining is also observed. Many investigators note that only the zymogenic region of the stomach is affected; the antrum/pyloric region or other parts of the gastrointestinal tract show any alterations. In an effort to describe the proliferative characteristics of these lesions, [^{3}H]-incorporation in the stomach of rhesus monkeys was investigated. Instead of finding labelled cells in the narrow isthmus region only, almost the entire mucosa below the most apical cell layers was labelled (Becker and McNulty, 1984).

Introducing the B1 cell

The immune system is of central importance for homeostasis and defence against hazardous agents that enter the body, e.g. via the gastrointestinal tract. Conventional B and T lymphocytes mature in bone marrow and thymus, respectively, where they undergo genomic rearrangement of their antigen receptors. They proceed through steps of positive and negative selection and finally the selected lymphocytes enter the periphery as mature cells, capable of interacting with antigen. In the periphery, mature lymphocytes continue to differentiate, depending on what signals they encounter. The development of the immune system has been extensively studied and reviewed elsewhere (Hardy and Hayakawa, 2001; Jameson et al., 1995; Kantor and Herzenberg, 1993; Zuniga-Pflucker and Lenardo, 1996). In contrast to conventional B cells (also called B2 cells) that develop in the bone marrow, a population of B cells that express CD5 (B1 cells) originate from fetal liver and omentum and form a distinctive peripheral B cell population, mainly present in the peritoneal and pleural cavities (Kantor and Herzenberg, 1993). This population is considered to be self-renewing and to produce most of the antibodies found in serum.

The antigen-bound B cell receptor complex transduces signals that may result in differentiation, proliferation and humoral immune responses. These signals are fine-tuned by an array of cytoplasmic and membrane bound signal transduction molecules that either amplify or dampen the B cell receptor signals during B cell development and during responses to self or foreign antigens (Tedder et al., 1997). There are two classes of surface molecules that appear to have opposing roles in modulation of the B cell receptor signal, reviewed in (Tsubata, 1999). Molecules that enhance signalling are the CD19/CD21 complex, CD38 and CD45 surface proteins. Thus, these positive regulators help to decrease the signalling threshold for the B cell receptor. On the other hand, among the negatively acting surface molecules that increase the signaling treshold are CD22, CD72 and FcγRIIB1 with their associated phosphatases SHP1 and SHIP (Tsubata, 1999). The inhibiting activity is probably crucial for avoiding B cell hyperactivity and autoimmunity.

The lack or reduction of the B1 population in the peritoneum has been reported in many different mouse models, most of which have deficiencies in molecules that modulate B cell receptor signalling. The differentiation of this lineage is disturbed, resulting in reduced or

absent peritoneal B1 cells, such as in Btk-deficient *xid* mice (Hayakawa et al., 1983) PKC-β-/- (Leitges et al., 1996), Vav-/- (Tarakhovsky et al., 1995; Zhang et al., 1995), Cr2 locus -/- (CD21/CD35, (Ahearn et al., 1996) and CD19-/- mice (Engel et al., 1995; Rickert et al., 1995). In addition, B1 cells are absent or decreased in mice deficient in the PI3 kinase subunit p85α, CD45, Igα, CD81 (which associates with CD19) as well as the transcription factors Oct-2 and Ailos, all factors that are important for Be cell receptor signalling or B cell differentiation (Pillai, 1999). B1 cells express high levels of the cytokine receptor for interleukin-5 and are stimulated to proliferate and differentiate by Il-5. Mice deficient for either the Il-5 receptor a chain (Yoshida et al., 1996) or the Il-5 itself (Kopf et al., 1996) do also show reduction of the B1 lineage in the peritoneum. In contrast, mice deficient in negatively acting molecules such as CD22 and SHP have an increased population of B1 cells in the peritoneum (reviewed in (Tedder et al., 1997). This is also seen in mice overexpressing CD19 (Tedder et al., 1997). Thus, the levels of B1 cells appear to be a very sensitive indicator of disturbed B cell receptor signal transduction.

Antibodies produced by B1 cells are mostly autoantibodies against self antigens and common bacterial antigens, and B1 cells are considered to be important for the first line defense against viruses such as Influenza (Baumgarth et al., 1999; Baumgarth et al., 2000; Ochsenbein et al., 1999), systemic bacterial infection (Boes et al., 1998) and nematode parasites (Paciorkowski et al., 2000). Interestingly, decreased resistance to infectious agents (e.g. Influenza virus) is one of the most sensitive effects of dioxin exposure in experimental animals (Burleson et al., 1996; House et al., 1990), but the role and effect on B1 cells after dioxin exposure remains to be elucidated.

Transgenic and knockout mice

The rat has traditionally been the tool of choice in toxicological research, due to a large amount of background data on e.g. clinical chemistry, pharmacological and kinetic parameters. However, there has been more genetical information available on the mouse, with more than 430 characterised inbred strains. Although transgenic rats have been developed, the mouse has been the species of choice for creating in vivo models of gene function.

The first paper on the successful creation of a transgenic mouse was published in 1982 (Palmiter et al., 1982b) and shortly thereafter was the first paper published describing an altered phenotype by overexpressing rat growth hormone in transgenic mice (Palmiter et al., 1982a). These models were the first examples of addition of an exogenous gene fragment (hence the word “transgenic”) that was successfully expressed in living mice and also being transferred to the next generation via the germ line. When these “conventional transgenics” are created, an exogenous gene fragment is injected into a fertilised mouse egg and thereafter introduced into a pseudo-pregnant foster mother. The exogenous gene fragment is randomly integrated into the genome, often in a head-to-tail orientation. Since the first reports, the use of transgenic animals has been a common tool in experimental biology and medicine.

The next step in manipulation of the mouse genome came in 1987 when the first successful gene disruption by homologous recombination in mouse embryonal stem cells (ES cells) was reported (Thomas and Capecchi, 1987). Two years later came the first report on mice in

which a disrupted gene was transmitted through the germ line (Thompson et al., 1989), so-called knockout mice. Thus, in this model system the role of a gene and its protein is indirectly studied by observing effects that are caused by a lack of the gene and its gene product. However, many knockout models have proven to be lethal, with embryonal death occurring early in gestation, making studies of the gene deficiency in the whole animal difficult.

Today, several more sophisticated strategies for adding or removing genes in mice and other species are available. Genes can be expressed or inactivated in a time-, organ- or cell type-specific manner. By carefully choosing the enhancer-promoter construct that regulates expression of an introduced gene the expression can be more specific. Transgenic expression can also be induced or shut off by the use of inducible systems, e.g. the tet on/ tet off system in which addition or removal of the drug tetracycline either turns on or shuts off expression of the transgene.

The use of the Cre-Lox system has made studies of otherwise embryonally lethal knockout models possible. When using this strategy, one mouse line is created by homologous recombination in which the gene fragment that shall be removed on each side is flanked by a recognition site (Lox P site) for the Cre-recombinase from the bacteriphage P1 (Lewandoski, 2001). This mouse line can then be crossed with a transgenic mouse line that expresses the Cre-recombinase in a time-, tissue-, or cell-specific manner. The Cre-recombinase specifically recognises the Lox P sites and removes the intervening sequence. Since mice expressing the Cre-recombinase are available for many cell types and developmental stages, this strategy of knocking out genes can be employed for most issues (Lewandoski, 2001).

More recently, the use of small interfering RNAs (siRNA) technology has boosted the hopes for yet another, more simple way of studying the deficiency of genes. The concept of RNA interference was presented only a few years ago and is based on a cellular machinery found in most (all) cells that degrades double-stranded RNA (Hannon, 2002; Hutvagner and Zamore, 2002). When short stretches (21-23 nucleotides) of complementary RNA are expressed in a cell (e.g. following direct transfection into the cell or following transcription from a transfected expression plasmid) or an organism (e.g. after feeding C. elegans siRNA expressing bacteria), the resulting siRNA/mRNA hybrid is degraded by this machinery. This has recently been reported to work in mice and rats (Hasuwa et al., 2002).

Aims of the present study

The dioxin/ Aryl hydrocarbon receptor is a ligand-activated transcription factor that mediates most (if not all) of the toxic effects of the group of highly potent environmental pollutants collectively called dioxins and PCBs. Although the high toxicity of these compounds has been known for more than three decades, the mechanism(s) of action behind the wide spectrum of effects is yet not known. Moreover, although the Ah receptor was cloned more than ten years ago, a physiological role or ligand has not unambiguously been identified. A mutant Ah receptor that shows constitutive transcriptional activity in cell cultures has been identified (McGuire et al., 2001). To study the functional role of this mutant in the context of a whole animal, transgenic mice were created that express this constitutively active Ah receptor (CA-AhR). The overall purpose of this thesis work was to characterize these mice and the more specific aims were:

- To establish homozygous lines of the transgenic mice and characterize expression and transcriptional activity of the CA-AhR in various tissues

- To study the biological effect of expression of the CA-AhR and to compare these to toxic effects observed in dioxin-exposed animals.

- To characterize biological effects that could possibly indicate a physiological role for the Ah receptor

Methodological considerations

The CA-AhR mice

The expression construct

CA-AhR demonstrates strong transcriptional activity in cell cultures (McGuire et al., 2001). Since the immune system is one of the most sensitive organ systems for dioxin toxicity, we chose to express the CA-AhR in lymphatic tissues. To achieve this, the coding sequence for CA-AhR was subcloned into an expression vector that previously has been shown to direct strong transgenic expression in both B and T cells (Fig. 6) (Bodrug et al., 1994). This construct consists of a strong promoter (SRα) which is a modification of the viral SV40 promoter (Takebe et al., 1988), and the Eμ enhancer of the Immunoglobulin heavy chain promoter (Banerji et al., 1983). In addition to these two regulatory elements, the construct contained a poly-adenylation signal as well as a splicing region from the SV40 large T gene (Fig. 6) for increased expression. The mice studied in this thesis are of the conventional transgenic type, i.e. an exogenous gene fragment was injected into fertilized eggs and integrated randomly into the genome. Thus, the endogenous, ligand-dependent AhR is still expressed and functional in these mice.

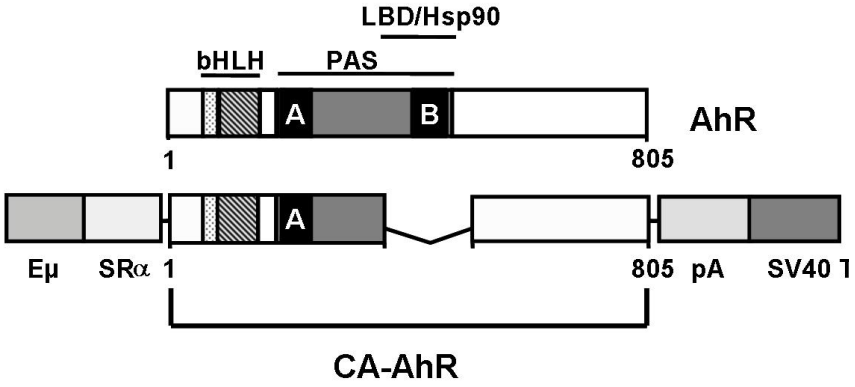

Figure 6. The CA-AhR and the Eμ-SRα expression construct shown together with AhR for reference.

Genetic background

Similar to most experimental systems, there are several pitfalls that need to be considered when creating and working with transgenic animals. Choosing an appropriate background strain can save a lot of work and time. At the time when the creation of the CA-AhR animals was being planned (1995), using fertilized eggs from a cross between mice of the C57Bl/6 (black) and CBA strains gave the highest success rate. Thus, when the resulting founder

animals were born they carried one allele of the C57Bl/6 background and one allele of the CBA background. The founder mice that were shown to have integrated the CA-AhR transgene were subsequently crossed with wild-type C57Bl/6. However, the progeny of this mating (F1 generation) did no longer have a defined genetic background since the contribution of C57Bl/6 or CBA alleles from the founder animal is likely to occur in a random fashion. Positive (heterozygous) F1 animals were mated with each other in order to generate homozygous animals with regard to the CA-AhR transgene, further mixing the genetic background.

A mixed genetic background could affect the response to TCDD treatment since different strains of mice express either a highly responsive AhR allele as examplified by the $AhR^{b/b}$ found in the C57Bl/6 strain, or a low-responsive AhR as examplified by the $AhR^{d/d}$ found in the DBA mouse strain (Poland and Glover, 1990). However, even in spite of different allelic origin, the Ah receptors in the C57Bl/6 and CBA strains are both of the highly responsive type (Poland and Glover, 1990). Inbred mouse strains also differ considerably regarding important factors such as spontaneous tumor incidence (see the Jackson Laboratory homepage:http://www.informatics.jax.org/external/festing/mouse/STRAINS.shtml).
Moreover, mice from different genetic backgrounds can differ with regard to haplotype of cell surface markers, creating problems in FACS analysis. This would also be a problem in functional immunological assays where the animals are challenged with infectious agents, since expression of different sets of MHC molecules (HLA in humans) would result in unequal efficiency of antigen presentation and subsequent unequal immune response. Thus, the results obtained may be the result of different genetic background, and not by the transgene or gene deletion, posing problems for interpretation of experimental results.

This can be solved by backcrossing the transgenic mice for at least ten generations with an inbred strain, e.g. C57Bl/6. Such back-crossing has recently been completed with the CA-AhR mice, but the present thesis is based on studies on CA-AhR and wild-type mice (not carrying the transgene) of the same mixed genetic background, a procedure that of course is not optimal. However, a mixed genetic background should in principle give rise to larger inter-individual differences compared to mice with a homogenous background, thereby decreasing the power in statistical analyses and concealing subtle differences. Today, technical improvements make it possible to make transgenic animals with a defined genetic background from start.

Analysis of several founder lines

Another pitfall is the possible disruption of vital genes which can be caused by the random genomic integration of the transgene. In other words, an interesting tumor phenotype could have nothing to do with the transgene *per se*, but rather be caused by the disruption of an important tumor suppressor gene, e.g. the retinoblastoma protein (Rb) or p53. The genomic integration of a transgene appears to occur in a strictly random fashion and the probability that the same integration would occur in two or three independent events is probably negligible. Therefore, to circumvent this problem, mice from more than one independent founder line should be analyzed. During the present work, three different CA-AhR founder lines were analyzed, two of which (animals of the Y8 and A3 founder lines) are described in the papers.

Genotyping

The presence or absence of the CA-AhR transgene in the mouse genome was verified in all mice by analysis of genomic DNA isolated from tail biopsies or the ear-clippings left after marking the mice. Semi-quantitative PCR analysis (Polymerase Chain Reaction) was used for routine screening of wild-type, heterozygous and established homozygous lines of animals. For this analysis, primers specific for the CA-AhR but also for the house-keeping gene β-actin were included in the same reaction tube. The presence of a β-actin PCR product verified that the genomic DNA template was of acceptable quality, helping to reduce the number of false negative results. The strength of the β-actin band could also function as an internal standard of the amount of template which had been added into the reaction tube, facilitating relative comparisons between the amounts of specific CA-AhR PCR products. The PCR screen is fast and sensitive, but in order to unambiguously discriminate between heterozygous and homozygous CA-AhR mice, Southern blot was performed. This procedure is more tedious than PCR but does not involve any amplification step, and is thereby more quantitative than PCR screening.

In the Southern blot procedure, genomic DNA was cut with specific restriction enzymes, precipitated and separated by electrophoresis in an agarose gel, blotted onto a Nylon filter by capillary transfer and covalently linked to the filter by exposure to UV-light. The filter was subsequently pre-hybridized and then hybridized overnight with a radioactivity-labeled (^{32}P) cDNA-fragment which was specific for the gene in question. After washing, the filters were exposed to X-ray film or analyzed by PhosphorImager analysis, with which quantification of the signal strength can easily be done.

Since CA-AhR animals have an intact endogenous AhR gene, this could be used as an internal loading control in the Southern blot procedure. The CA-AhR coding sequence does not contain any introns. Thus, by choosing a restriction enzyme (NcoI) that cuts several exons apart, the result was a shorter fragment from the intron-less CA-AhR cDNA and a longer fragment from the endogenous, intron-containing AhR gene.

Analysis of CA-AhR and target gene expression

RT-PCR

The initial screen for CA-AhR expression was done by RT-PCR, which is PCR analysis on cDNA templates which have been reversely transcribed from RNA, using reverse transcriptase. The expression of the CA-AhR could be discriminated from the endogenous AhR by different sizes in the gel electrophoresis analysis. Analogous to the problems involved with conventional PCR, quantitative interpretation of RT-PCR results should be done with caution. If the primers and protocol are carefully designed, RT-PCR is a very sensitive method to assess whether a transcript is present or not, and semi-quantitative RT-PCR (including a house-keeping gene) can give an estimate of large variations in expression levels. However, in order to give accurate quantitative results, special approaches need to be taken, such as extensive dilution series, the inclusion of competitor sequences or using the

recently developed technique of Real-Time PCR. Instead of doing this, we turned to the method of Northern (RNA) blotting.

Northern (RNA) blot

The procedures of Northern (RNA) blotting are analogous to Southern (DNA) blotting. Depending on which genes were going to be analyzed, the samples contained either total-RNA or poly-A RNA enriched by oligo-T-linked magnetic beads (Dynabeads, Dynal, Oslo). The endogenous Ah receptor and the CA-AhR were expressed at low levels and could only be detected when poly-A RNA was analyzed. In most of the analyses, a conventional formamide-containing hybridization buffer was used. Even though poly-A RNA was being analyzed, the use of that hybridization buffer sometimes required long times for film exposure (3-4 weeks). More recently, a commercial hybridization buffer was used (Ultrahyb, Ambion), which actually reduced the exposure time several-fold. Radioactively labeled probes could fairly gently be stripped away by boiling in 0.1% SDS, followed by cooling in room temperature. By this procedure, the filter could be hybridized again with a different probe, thereby detecting expression of other genes in the same samples. This way, expression of CA-AhR, target genes and house-keeping genes such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase), could be analyzed on the same filter. Up to nine different hybridizations were successfully done with the same filter. In all quantifications of Northern blot experiments the difference in loading input was corrected for by normalization against GAPDH.

In situ hybridisation and Immunohistochemistry

The RNA samples used in the RT-PCR, Northern blot and suppressive subtractive hybridization (SSH, see below) analyses were isolated from tissue homogenates and the results therefore reflect the expression levels in the organ or part of the organ as a whole. To study expression levels in individual cell types, in situ hybridization and immunohistochemistry can be used for expression studies at the mRNA and protein levels, respectively. Since the CA-AhR is a deletion mutant of the endogenous AhR (see above) the expressed mRNA species will have homologous sequences (except for the deletion), which make design of a probe for in situ hybridization more difficult. In Northern blot analysis, the mRNA species are discriminated by their different sizes, but this criterion cannot be used in intact tissue sections. However, a probe that spans the junction of the PAS B deletion in the CA-AhR could in principle work (Fig. 7). If this probe would be short enough (50-300 nucleotides) it would be washed away from the endogenous AhR mRNA due to lower

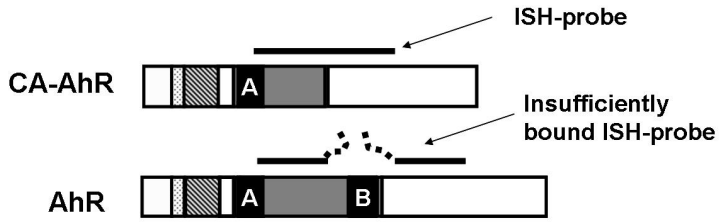

Figure 7. Principle of probe for In situ hybridization of the CA-AhR deletion mutant

affinity caused by the non-homologous overhangs. This approach was used, first by using non-radioactively digoxygenin (DIG)-labeled and radioactively labeled (^{33}P) DNA-oligonucleotides (around 50 nucleotides) as probes and later with an alternate protocol employing radioactively labeled (^{33}P) RNA-probes. In these experiments, expression of CYP1A1 was distinctly observed in the stomach of the CA-AhR animals (data not shown), and optimization of the protocol for detection of CA-AhR is currently ongoing.

The presence of the endogenous AhR poses problems also for specific detection of CA-AhR by antibodies. The best available antibodies raised against AhR recognize both the AhR and the CA-AhR (Fig. 1C in paper I). Thus, instead of direct detection of the CA-AhR protein in tissues, some alternative, indirect approaches were used. In the interpretation of stomach sections stained with the AhR and CYP1A1 antibodies, we took advantage of the fact that the CA-AhR shows constitutively nuclear localization compared to the endogenous AhR which shows even distribution both in the cytoplasmic and nuclear compartments (McGuire et al., 2001), and combined observations of subcellular Ah receptor localization with CYP1A1 staining (see Results).

Some of these obstacles could have been overcome with a different design of the expression construct. Addition of six histidines to the CA-AhR could have facilitated the use of specific His_6-antibodies. However, at the time the mice were created, this was believed to reduce expression efficiency and it could possibly also influence the function of the CA-AhR. Another approach could have been the addition of the coding sequence for an easily detectable marker protein, e.g. the enzyme β-galactosidase (LacZ) or green fluorescent protein (GFP) (Hadjantonakis and Nagy, 2001), regulated by a so-called internal ribosomal entry site (IRES) (Mountford and Smith, 1995). By this strategy, the translation of the gene of interest (e.g. CA-AhR) is initiated at the 5'-cap of the mRNA and translation of the marker protein is initiated by the IRES. This results in faithful coexpression of the gene of interest and the marker protein (Mountford and Smith, 1995), making detection (albeit indirect) of the gene of interest possible without addition of any exogenous tag to the protein. An alternate approach to this problem would be selective dissection of tumor tissue (by e.g. laser capture technology) with subsequent analysis of CA-AhR and target gene expression.

Suppressive Subtraction Hybridisation (SSH)

Suppressive subtraction hybridization is a powerful method for the identification of differentially expressed genes of low abundance. In this procedure, cDNA synthesized from a target (i.e. treated or pathological tissue used as source of RNA) is subtracted against cDNA from a control source (untreated or normal tissue) and the remaining fragments are selectively amplified for downstream applications. The procedure used in paper III has previously been published (von Stein, 2001), but will briefly be described here.

The starting material was poly-A RNA isolated from a control source (C) and from a target source (T, Fig. 8A). This RNA was reversely transcribed to cDNA by the use of oligo-dT primers (that bind the poly-A tail of mRNA) containing the four bases long recognition site for the restriction enzyme *RsaI*. This enzyme cuts on average once in 256 base-pairs and after cutting the cDNA, the subsequent pool of cDNA contained fragments ranging from approximately 100-400 bases (Fig. 8B). This pool was split into three parts, of which two

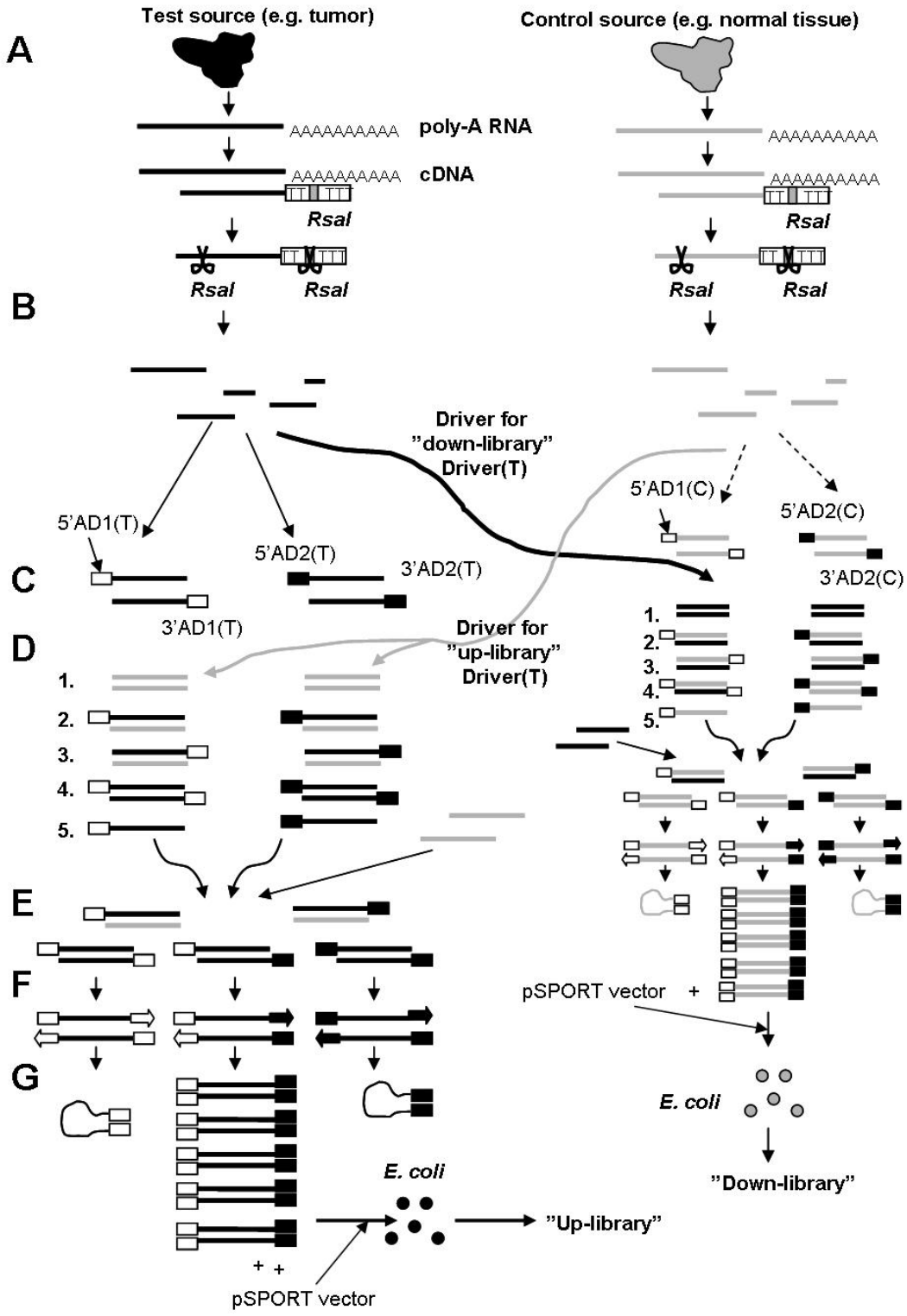

Figure 8. The principles of suppressive subtractive hybridization (SSH). Please refer to text for understanding

were ligated to two different 40 bases long adaptors (AD1 and AD2), either at the 5' or 3' end of the digested cDNA (Fig. 8C). The third part of the tester pool of cDNA was used as a

"driver" in parallel analysis of down-regulated genes (see below). In separate tubes, the AD1(T) and AD2(T)-ligated fragments were denatured and hybridized against an excess (approx. 20:1) of *RsaI*-digested, but unligated cDNA from the control source, the "Driver(C)". The resulting hybrids were of five different types (see Fig. 8D): **1.** Driver(C):Driver(C); **2.** 5'AD1(T):Driver(C); **3.** 3'AD1(T):Driver(C); **4.** 5'AD1(T):3'AD1(T) and **5.** non-duplexed 5'AD1(T) or 3'AD1(T). The non-duplexed fragments (5.) represented genes that were overexpressed in the target. The corresponding duplexes were formed with the AD2-ligated fragments (Fig. 8D).

The pools of AD1(T):Driver(C) and AD2(T):Driver(C) hybrids were mixed together and additional, freshly denatured Driver(C) was added (Fig. 8E). The only new hybrids that formed were those between the previously non-duplexed 5'(or 3')AD1(T) and AD2(T), i.e. no. **5.** in Fig. 8D. The overhanging ends of the hybrids were then filled in by a DNA polymerase, creating three different hybrids with either adaptor AD1 or AD2 at the 5' and 3' ends (Fig. 8F). This pool of hybrids was then subjected to PCR amplification with selective primers with matching sequences within the AD1 and AD2 adaptors (Fig. 8G). In addition to being complementary to the primers, the adaptors were designed to form "intra-fragment" duplexes if the fragment contains the same adaptor in both the 5' and 3' end (i.e. 5'AD1-AD1 3', Fig. 8G). These "intra-fragment" loops were more stable than the AD:primer hybrid and were not amplified. The pool of selectively amplified fragments constituted the subtracted "up-library" and was subcloned into the SalI-NotI restriction site of the pSport plasmid vector. Exactly the same procedure was performed to generate the subtracted "down-library", but *RsaI*-cut cDNA from the control source was instead ligated to the adaptors, generating AD1(C) and AD2(C) fragments that were hybridized with an excess of *RsaI*-cut, but unligated target cDNA as driver Driver(T).

The "up- and down-libraries" were used to transform bacteria of an E.coli strain. These bacteria were grown on 22 cm^2 plates (4-5000 colonies/plate) and positive colonies were then objectively and randomly picked and transferred to a 384 wells-plate by a robot equipped with an optical device. The bacterial clones were cultured overnight and inserted DNA fragments of these clones were amplified by PCR in 384-well plates, using the primers corresponding to the adaptors AD1 and AD2. The resulting PCR products from the "up-library" and the "down-library" were spotted onto a nylon-filter in duplicates, samples from "up" always above the "down" spots. The spotting pattern also included a spot of genomic DNA for orientation and normalization purposes. Between 12 and 24 identical filters were spotted at the same time.

Pairs of filters were subsequently hybridized with subtracted and amplified ^{33}P-labeled cDNA fragments, representing the material for generation of "up-library" and "down-library". After washing, the filters were subjected to PhosphorImager analysis. The resulting computer images were colored by red (for the "up-library") and green (for the "down-library") using software L Process (v.2.0). When these two images were merged, red spots indicated positive "up-clones", green spots indicated positive "down-clones" and yellow spots indicated equally expressed clones. PCR-products representing the different positive "up-" or "down-" regulated clones were sequenced and identified by a conventional BLAST-search.

SSH vs. Microarrays

Compared to the established gene expression methods of "array-type", the SSH is quite labor intense. However, there are several advantages. First of all, it is an open system: any gene (including previously unidentified) that is differentially expressed could in principle be detected, in contrast to the list of genes defined beforehand in the array-analyses. Moreover, rare transcripts, that may be undetected in the array-analysis, are enriched by this method. In addition, since bacterial clones and PCR are generated that represent the individual genes of the "up- or down-libraries", cDNA for use as probes in subsequent verification experiments are very easily obtained. However, the SSH procedure described above takes two to three weeks to complete, which (unlike array analyses) makes it unsuitable for screening a large number of samples. In addition, it does not provide any quantitative information about differences in expression levels. Thus, an optimal approach would be to use SSH for the initial identification of differentially expressed genes and to use microarray for subsequent analysis of those genes.

Using CYP1A1 as a marker of transcriptionally active CA-AhR and Ah receptor

Constitutive transcriptional activity of the CA-AhR was demonstrated by analyzing expression of the Ah receptor target gene CYP1A1. The use of this marker was rewarding, since there is practically no basal expression of this gene but rapid and strong induction when cells or tissues are exposed to low levels of Ah receptor ligands such as TCDD (Figs. 2A-C, paper I and figs. 3A and B, paper III). Expression of this sensitive marker could easily be shown by RT-PCR screening and in stomach sections by immunohistochemistry (Figs. 4G-H, paper I). In addition, CYP1A1 was used as a marker when the level of transcriptional activity of the CA-AhR was compared to the transcriptional response evoked by exposure of wild-type animals for different doses of TCDD (Figs. 2A-D, paper I).

TCDD exposure of the mice

TCDD exposure of cultured cells and wild-type animals was used as positive controls for Ah receptor activation. In the animal experiments, we chose oral exposure by gavage, partly since we wanted to get a strong activation response in the stomach by local uptake of TCDD, but also since it is the most relevant route of exposure for humans, i.e. via the food. After some initial pilot experiments, we decided to expose six months old wild type females to increasing doses of TCDD, ranging from 0.003 to 3 μg/kg body weight. Corn oil was used as vehicle. Vehicle only was given to the heterozygous and homozygous CA-AhR animals that would be compared to TCDD treated wild-type mice. The animals were sacrificed three days later (72 hours), a time long enough for TCDD to become evenly distributed in the body, but short enough to avoid too much excretion. However, CYP1A1 expression levels in the stomach of the CA-AhR mice exceeded those observed in the highest dose group in this experiment (see Results below). Therefore subsequent TCDD exposures used doses of 10 μg/kg bodyweight for 24 hours.

FACS – Fluorescence Activated Cell Sorter

FACS analysis was initially developed for sorting cells more than 30 years ago (Herzenberg and De Rosa, 2000), but was quickly developed for use in a number of different applications. Together with the development of monoclonal antibodies (mAbs), FACS analysis has been central for the evolution of modern immunology (Herzenberg and De Rosa, 2000). One of the most common FACS applications, analysis of lymphocyte populations by staining cells with different combinations of fluorochrome-conjugated antibodies, was used in paper IV.

In the FACS analysis, single-cell suspensions of cells are labeled with antibodies conjugated to different fluorochromes. The stained cells are passed, one cell at a time, in a very narrow beam through one or more laser(s) at high speed. Depending on the wavelength of the lasers, the fluorochromes become excited, and the following emission of light is detected. Live lymphocytes can be defined according to their forward (size) and side (granularity) scatter. This area is marked and called a "gate". In the subsequent analyses, only cells that fulfill the forward and side scatter criteria for live lymphocytes are usually shown; the other cells are "gated" out. Gates can also be applied to a cell population (of live lymphocytes) that is defined by the expression of a certain combination of surface markers (e.g. gate R1 in Fig. 4A, paper IV)**,** and this subpopulation can then be analyzed with regard to the expression levels of a third marker (e.g. Fig. 4B in paper IV). The results of the FACS analyses are usually presented as percent of the total number of cells included in that gate.

For each combination of stainings, a large number of cells ("events") is usually analyzed. In paper IV, between 30 000 and 60 000 events were recorded for each staining and tissue. The FACS machine that was used in paper IV was equipped with one laser, making triple staining of the cells possible and thus, five parameters per cell was recorded. Today, there are FACS machines equipped with three lasers, making simultaneous analysis of up to 11 different colors (and surface markers) possible (Herzenberg and De Rosa, 2000). A FACS machine can be used for numerous other applications such as cell sorting and analysis of efficacy of cell transfection by co-transfection of e.g. GFP (Misteli and Spector, 1997). Moreover, FACS can be used in analysis of the cell cycle and apoptosis (Ormerod, 2002).

Results and Discussion

CA-AhR founder lines

Out of 33 live born pups, five animals were found to carry the CA-AhR transgene and constituted the CA-AhR founder animals. After consecutive rounds of breeding and analyses of CA-AhR and CYP1A1 expression by RT-PCR, three founder lines, designated Y4, Y8 and A3, were chosen for further studies. The first founder line that was found to be homozygous with regard to the CA-AhR was the Y8, and most of the subsequent analyses were done on those mice. Homozygous mice of the A3 line were also established relatively easily and used in several of the experiments (Papers I-IV). In contrast, a line of homozygous Y4 mice was difficult to obtain. The reason for this is presently unknown. Expression of the CA-AhR did not seem to differ markedly between these lines (data not shown). In the Y4 line, the CA-AhR construct could in principle have been integrated into an essential locus, leading to mortality in homozygous Y4 CA-AhR mice which then entirely would lack expression of that gene. This question warrants further investigation.

Sex ratios and bodyweight

The homozygous CA-AhR animals were fertile, and mating heterozygous Y8 and A3 animals yielded homozygous, heterozygous and negative progeny in approximately the expected Mendelian 1:2:1 ratio. An increased frequency of girls has been reported from several human populations exposed to TCDD or other Ah receptor ligands (Mocarelli et al., 2000; Ryan et al., 2002). However, homozygous CA-AhR did not show any significant alterations in the sex ratio compared to wild-type mice. In fact, in CA-AhR mice of the Y8 and A3 founder lines, the frequency of males were 50% and 57%, respectively, compared to 55% in wild-type mice.

Exposure of experimental animals to high doses of TCDD results in a lethal wasting syndrome which is characterized by decreased food intake and dramatic weight loss (Pohjanvirta and Tuomisto, 1994). Compared to wild-type animals, the body weight of male homozygous CA-AhR animals of the A3 founder line was decreased, beginning at 27 weeks of age (Fig. 9). Surprisingly, at eight and ten weeks of age homozygous Y8 males showed higher body weights than wild-type mice. This difference disappeared with increasing age and Y8 males 37 weeks or older showed decreased body weight (Fig. 9). Although observed long before the A3 animals become moribund, the decreased body weight is probably associated with the increased mortality discussed above. The reason for the increased body weight observed in young Y8 males is presently unknown.

In analogy with observations in TCDD-exposed experimental animals (Fletcher et al., 2001; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982), the weights of thymus and livers were decreased and increased, respectively, in the CA-AhR mice (Figs. 2E and F, paper I). In addition, several other organs from the CA-AhR mice showed altered relative weight compared to wild-type animals (data not shown). Altered organ weights are only crude

indicators of any pathological alterations and should be complemented by a histopathological evaluation. Such studies are currently ongoing.

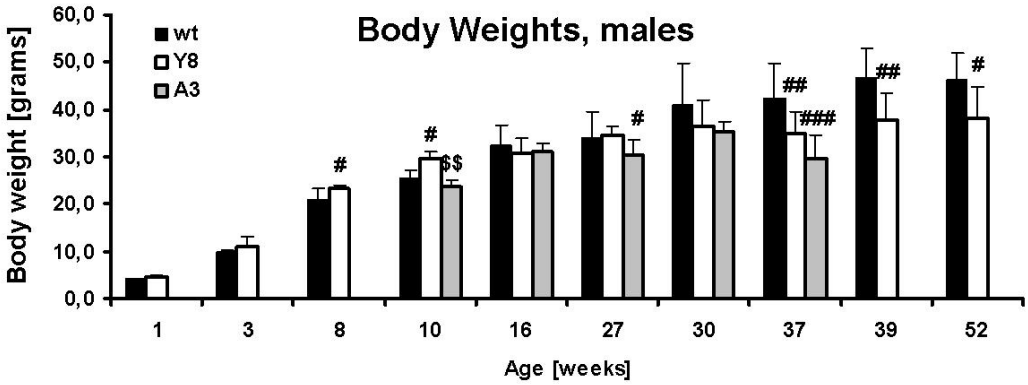

Figure 9. Average body weights of wild type and CA-AhR mice of the Y8 and A3 founder lines. At least four animals were analyzed in each group. #, ##, ## denotes $p<0.05$, 0.01 or 0.001, respectively, as assessed by two-tailed student's t-test.

Increased mortality

A true mortality study has not been performed due to ethical reasons, but CA-AhR mice were found to show increased mortality. Homozygous male mice from both the Y8 and the A3 founder lines were found dead in their cages at an earlier age than female mice (Fig. 2G, Paper I), and A3 animals showed the highest mortality when these two founder lines were compared (Fig. 2G, Paper I). These deaths were not preceded by any long-term apparent clinical signs of illness. When body weights of older A3 animals were recorded by daily intervals, a sudden drop in body weight (by 5%) could occasionally be noted (data not shown). Within 24 hours, those animals became moribund and were sacrificed. Thus, the process leading to death appears to be very rapid. Although the increased mortality correlates well with the development of stomach tumors, the ultimate cause of death is presently unknown.

Expression and transcriptional activity of the CA-AhR

As expected (see "Methodological considerations"), CA-AhR was expressed in lymphatic organs, e.g. in thymus, spleen, lymph nodes, enriched T and B cells and bone marrow. Moreover, as demonstrated in paper I (Fig. 1D) and in paper IV (Figs. 1A and B), the CA-AhR was also expressed and transcriptionally active (as assessed by CYP1A1 induction) in e.g. liver, lung, muscle, skin, brain, heart, kidney (Fig. 1D, paper I) and the entire gastrointestinal tract (Fig. 3P, paper I).

This wide-spread expression pattern was quite surprising, considering the low level of extra-lymphatic transgene expression demonstrated in a previous study using the same expression construct (Bodrug et al., 1994). However, when this paper was scrutinized, it was found that expression of the house-keeping gene was barely detected in the non-lymphatic organs,

indicating considerable less sample-input from these organs (Bodrug et al., 1994). This non-restricted expression pattern could be caused by the strong SRα promoter used in this expression construct (Takebe et al., 1988), but also the Eμ enhancer has been shown to direct expression in other organs (Jenuwein and Grosschedl, 1991).

Since the CA-AhR show constitutively nuclear localization compared to the evenly distributed endogenous AhR (McGuire et al., 2001), the subcellular staining pattern resulting from staining with the AhR antibody was analyzed. In contrast to the evenly distributed AhR in glandular stomach of wild-type animals (Fig. 4M, paper I), tumor tissue from CA-AhR mice showed a predominant nuclear staining (Fig. 4O, paper I), consistent with a transcriptionally active protein. In combination with the distinct CYP1A1 expression in the stomach tumors (Fig. 4I, paper I), it was concluded that the CA-AhR is expressed and transcriptionally active in this tissue.

The fact that homozygous CA-AhR animals showed higher CYP1A1 mRNA levels compared to heterozygous mice (Figs. 2A-C, paper 1), and the induction of other established target genes such as CYP1A2 and AhRR (Figs. 1D, 2B and 1E in paper I, respectively) and NMO1 (Fig. 1A, paper III) provided compelling evidence for the transcriptional activity of the CA-AhR. However, comparison of the expression levels of CA-AhR and the target gene CYP1A1 revealed that these did not correlate (Figs. 1D, paper I). For instance, liver, muscle, skin and heart showed considerable higher CYP1A1 expression compared to thymus and spleen even though the CA-AhR was expressed at a higher level in these two latter organs. This observation indicates that there are other, tissue-specific factors which influence expression of the CYP1A1 gene.

When the transcriptional activity of the CA-AhR was compared to wild-type animals exposed to different doses of TCDD, the CYP1A1 induction levels in homozygous CA-AhR was similar to wild type animals treated with 3 and 0.3 μg TCDD/kg bodyweight in thymus and liver, respectively (Figs. 2A and B, paper I). Thus, the activity of the CA-AhR in these tissues corresponds to exposure to a low dose of TCDD. The CA-AhR activity in the stomach exceeded the highest dose given in this experiment (Fig. 2C, paper I), but subsequent experiments demonstrated that it corresponded to a TCDD dose of 10 μg/kg body weight or higher in the glandular part of the stomach (Fig. 2D, paper I). The differences in CYP1A1 induction levels by the CA-AhR could possibly be explained by cell type-specific expression of the CA-AhR. So far, we have demonstrated epithelial expression and activity of the CA-AhR in the glandular stomach. It is possible that the CA-AhR is expressed in other organs in non-epithelial cells that may be not as permissive as the stomach for CYP1A1 induction. Experiments to resolve this question are being planned.

Stomach tumors

Even though the CA-AhR animals did not show any apparent clinical signs of illness, they were occasionally found dead in the cage (see section on increased mortality, above). When necropsy was done, large cysts of different colors were found on the stomach. Over time, several pathologists experienced in work with experimental animals and/or specialists in gastrointestinal pathology have contributed with histopathological assessments of the stomach

lesions found in the CA-AhR animals. The descriptions have ranged from “not tumors”, “congenital malformation”, to “gastric adenocarcinoma”. The difficulty in putting a label on these lesions indicated that they showed a rare combination of characteristics. For reasons discussed at the end of this section, the term “tumor” was chosen for glandular structures penetrating into the submucosa and beyond.

A more systematic collection of stomach tissue from wild-type and CA-AhR animals was done. Analysis of these samples is described in papers I and II and will here be briefly summarized. The first macroscopically visible lesions were small cysts filled with clear content found in the minor curvature of the stomach (Fig. 3A, paper I). In older animals, cysts were more numerous and varied in color from clear, white and beige to brown and black (Fig. 3C, paper I and Fig. 1A, paper II). At this stage, dark spots could also be seen externally in the stomach wall (Fig. 1A, paper II). In animals around 12 months of age, the cystic stomachs were found to adhere to surrounding organs, such as liver, pancreas, spleen and fat (Fig. 3D, paper I and Figs. 2H and I, paper II). On the inside of the stomach, the so-called limiting ridge separates the squamous epithelium of the forestomach from the glandular part of the stomach. This border was substantially enlarged in the CA-AhR animals, and bleeding was occasionally observed in the mucosa (Fig. 1D, paper II).

Cystic dilatation and penetrating tumoral glands

The earliest microscopical alterations (at 9 weeks of age) observed in the CA-AhR glandular stomach were cystic dilatations of basally located glands (Fig. 1H, paper II). Starting at 10 weeks, isolated cystic glands were found to penetrate through the submucosa, the muscularis propria and reaching the subserosa (Figs. 3F, G, paper I and Fig. 1F, paper II). These tumors were found to be actively proliferating as demonstrated with staining for the proliferation marker PCNA (proliferating cell nuclear antigen; Fig. 1G, paper II). This penetration was not caused by herniation since the glands were not surrounded by bulging muscularis mucosa, but instead by connective tissue as demonstrated by the van Gieson stain (Fig. 3H and I, paper I). In addition, in 10 weeks old CA-AhR animals foveolar hyperplasia in the mucosa and expanding glandular structures within the limiting ridge were found (Figs. 1F and I, paper II and Fig. 3F, paper I).

In older animals, tumors were found to penetrate into the muscularis propria at several locations, resulting in a substantially thickened stomach wall with multiple cysts of varying size (Fig. 2A, paper I). This could sometimes give a quite bizarre overall appearance (Fig. 3J, paper I). The tumors were found to be composed by highly differentiated gastric cells, such as foveolar and parietal cells as well as cells of cardio-pyloral type (Figs. 2C, D and L, paper II), but metaplastic epithelium (squamous metaplasia) was also observed (Fig. 2G, paper II). In addition, many tumors were surrounded by large amounts of connective tissue, vessels as well as well-organized lymphatic foci and fatty tissue (Fig. 3K, paper I and fig. 2F, paper II). Lesions with these characteristics are classified in human pathology as hamartomatous tumors. Although tumor-laden stomachs were found to adhere to adjacent organs (Figs. 2H and I, paper I), penetration into these organs or metastases to other sites have to date not been found.

Intestinal metaplasia

The columnar tumor cells expressed neutral mucins (which is normally synthesized by foveolar cells) as demonstrated by PAS-staining (Fig. 2M, paper II). Moreover, these cells expressed acid mucins, not including sulphomucins, as demonstrated by positive staining for Alcian Blue, pH 2.5 (Fig. 3M, paper I and Fig. 2J, paper II) and negative staining for High Iron Diamide (data not shown). Acid mucins are normally not expressed in the stomach. These findings therefore demonstrate intestinal metaplasia in the stomach tumors of the CA-AhR mice.

Influence of sex and founder line

A general assessment of the overall severity of the stomach tumors was presented in paper I (Fig. 5), demonstrating a difference between heterozygous and homozygous animals as well as between males and females. In a more quantitative description, hematoxylin-eosin (HE) stained stomach sections from both sexes of wild-type and two independent CA-AhR founder lines of different ages (3 to 52 weeks of age) were microscopically evaluated for several parameters. These included glandular cysts found in the mucosa or muscularis propria or the presence of squamous metaplasia, lymphatic foci, goblet cells or glands within the limiting ridge (Paper II). Male mice were found to develop cysts in the mucosa and showed tumors in the muscularis propria at an earlier age than female mice (Figs. 3A and B, paper II). Moreover, a difference between male mice from the Y8 and A3 founder lines was also observed, both with regard to the presence of mucosal cysts and tumors (Figs. 3C and D, paper II) and relative stomach weight (Fig. 3E, paper II). This difference between sex and founder lines is in good agreement with the differences observed with regard to mortality (Fig. 2G, paper I). Apart from isolated glandular dilatations and occasional goblet cells found in older animals (Table 1, paper II), none of the lesions described above was found in wild-type animals.

Decrease in parietal cells

During the evaluation of the HE-stained stomach sections, it was noted that the fraction of parietal and chief cells seemed to be reduced in CA-AhR animals. However, difficulties in obtaining an antibody specific for mouse parietal cells precluded any firm conclusions. By serendipity, a surrogate marker for parietal cells was discovered (described and shown in paper II, Figs. 4A-D). When stomach sections were stained with this marker, a distinct decrease of the parietal cell region in CA-AhR mice was observed (Figs. 4E and F, paper II). Decreased expression of the β-subunit of the parietal cell marker H^+/K^+ ATPase (Fig. 4G, paper II) further substantiated this observation. Moreover, quantification of the parietal/chief cell region in HE-stained sections also demonstrated a decrease of this region in 16 weeks or older males of the A3 founder line and in 52 weeks old males and females of the Y8 founder line (Fig. 4H and I, paper II), again demonstrating a difference between the founder lines.

In addition, exposure of wild-type animals to TCDD resulted in expansion of the zone of proliferating cells normally found in the narrow isthmus-region of the stomach (Fig. 4D, paper I) to include the entire parietal-chief cell region (Fig. 4E, paper I). This suggests that not only the CA-AhR, but also the ligand-activated AhR can affect the proliferative status of cells in the gastric mucosa.

Benign or malignant?

According to Dorland's illustrated medical dictionary (1985), the term "tumor" describes "a new growth of tissue in which the multiplication of cells is uncontrolled and progressive, called also neoplasm". Under the entry "neoplasm" in the same dictionary, one can read "Malignant neoplasms are distinguished from benign in that the former show a greater degree of anaplasia and have the properties of invasion and metastasis". The term "anaplasia" is explained by "a loss of differentiation of cells (dedifferentiation) and of their orientation to one another and to their axial framework and blood vessels, a characteristic of tumor tissue". Finally, under the heading "cancer" one can read: "a cellular tumor the natural course of which is fatal" and "cancer cells, unlike benign tumor cells, exhibit the properties of invasion and metastasis and are highly anaplastic" (1985).

Considering these definitions, the lesions observed in the glandular stomach of the CA-AhR mice display some really paradoxical characteristics. The cells that constitute the penetrating glands are highly differentiated and may even at a small scale be organized like gastric mucosa of zymogenic type (Fig. 2C, paper II) or of antral/pyloral type (Figs. 2D and L, paper II). The columnar cells retained the capability of secreting neutral mucins (Fig. 2M, paper II), and no signs belonging to commonly accepted criteria for dysplasia or malignancy were found. Despite all this, "uncontrollably" proliferating cells from the gastric mucosa penetrated into the submucosa, muscularis propria and reached the subserosa; squamous and intestinal metaplasia was observed; stomachs adhered to adjacent organs and increased mortality was observed in the CA-AhR animals. Thus, highly differentiated cells (indicating non-tumor), show uncontrolled and progressive proliferation (indicating neoplasia/tumor) with properties of invasion that most likely contribute to the increased mortality (indicating cancer/malignancy). Until further properties of these cells have been evaluated (such as the potential for ectopic growth in immuno-deficient nude mice), we have conservatively used the neutral term "tumor" for describing cells that penetrated into the submucosa and beyond.

As described in the introductory section, there exist several reports on the effects of Ah receptor ligands in the gastrointestinal tract. Notably, all of the described effects show the same characteristics after exposure to commercial PCB mixtures (Aroclors) (Allen et al., 1974; Allen and Norback, 1973; Becker et al., 1979; McConnell et al., 1979; Morgan et al., 1981; Ward, 1985); the dioxin-like 3,4,3'4'-TCB (PCB77) (Becker and McNulty, 1984; McNulty et al., 1980; Silverman et al., 1979; van den Berg et al., 1988); commercial mixtures of polybrominated biphenyls (Gupta et al., 1983; Lambrecht et al., 1978) or TCDD-like dibenzofurans (Brewster et al., 1988; Hebert et al., 1990; McNulty et al., 1981). This included a marked hyperplasia of the surface mucous cells and a substantial decrease in the number of parietal and chief cells as well as intestinal metaplasia. Cystic dilatations are seen in the deep gastric glands and glandular structures penetrate into the submucosa, often associated with foci of lymphatic cells. Only the zymogenic region of the stomach is affected; the antrum/pyloric region or other parts of the gastrointestinal tract show no alterations. Analysis of proliferating cells by [^{3}H]-incorporation demonstrated that in the stomach of rhesus monkeys almost the entire mucosa was labelled in contrast to control animals where only cells in the narrow isthmus region were labelled (Becker and McNulty, 1984). However, the

tumors observed in the CA-AhR mice did not only penetrate the submucosa, but also the muscularis propria reaching the subserosa.

Dysregulated gene expression in the glandular stomach – osteopontin

Suppressive subtraction analysis, SSH

The search for dysregulated genes causing the tumors was done with an unbiased approach. Thus, even though several genes and their protein products have been shown to be associated with the development of stomach tumors and cancer in man and in experimental animals, we chose not to evaluate expression and/or function of these one by one. Instead, we turned to the method of suppressive subtraction hybridization (SSH; discussed above). In order to detect relatively early changes in gene expression, glandular stomachs from 13 weeks old male CA-AhR mice were compared to wild-type males (Paper III). To this date, two rounds of analysis have been done on the subtracted material from the 13 weeks old mice. In these two analyses, a total of 37 clones representing upregulated and 16 clones representing genes down-regulated in the CA-AhR stomachs were found. Several of the genes were detected in both of the analyses and some genes were represented by more than one clone in each round of analysis. Of the upregulated genes, 11 were enzymes; 6 were associated with inflammation and cancer; 3 associated with energy metabolism; 10 genes were ascribed other functions**,** and 7 clones represented hypothetical proteins. Of the down-regulated genes, 4 were enzymes; 4 were associated with cancer and inflammation; 1 with energy metabolism; 5 had other functions and 2 clones represented hypothetical proteins. Several of these genes that may be of specific interest for the etiology of the stomach tumors are currently being investigated and will be published elsewhere.

Decreased osteopontin expression in the CA-AhR mice

Analysis of one of the down-regulated genes, osteopontin, is described in paper III. Northern blot analysis of RNA from 3.5 months old CA-AhR mice from both the Y8 and A3 founder lines demonstrated significantly increased expression levels of CYP1A1 as well as NMO1 (Fig. 1A, paper III), which, in addition to CYP1A1 represents a well-established Ah receptor target gene (Robertson et al., 1986; Vasiliou et al., 1994). Expression of osteopontin was decreased in both of these founder lines as well as in 9 months old CA-AhR males of the Y8 line (Fig. 1A and B, paper III). This decrease was observed in 58 days or older Y8 males (Fig 1C, paper III). Moreover, osteopontin expression seemed to be decreased in other organs of the CA-AhR mice, such as brain and kidney (Fig. 2A and B, paper III).

Decreased osteopontin expression is dependent on functional AhR and Arnt

Cultured wild-type hepatoma cells exposed to 0.001 to 10 nM of TCDD for 24 hours showed strong induction of CYP1A1 and NMO1 but no discernable effect on osteopontin expression (Fig. 3A, paper III). However, when these cells were exposed to the highest TCDD concentration used in the dose-response experiment (10 nM) for various length of time, a

rapid, but transient decrease in osteopontin expression was observed (Figs. 3B and C, paper III). In contrast, mutant, dioxin-resistant hepatoma cells (Hankinson, 1994) that either express substantially reduced Ah receptor levels (the C12 mutant) or a functionally defect Arnt protein did not show any reduced expression levels of osteopontin (Fig. 4A and B, paper III). Since the Ah receptor signaling pathway is non-functional in these cells as demonstrated by the absence of the TCDD-dependent CYP1A1 induction response (Fig. 4A, paper III), the present data suggest that the decrease in osteopontin expression observed in the CA-AhR mice and in TCDD exposed cells is dependent on both the Ah receptor and Arnt.

Several studies have reported an association between increased expression of osteopontin and malignant and metastatic progression of several different human tumors, including cancers of the breast, lung, bladder, kidney (Brown et al., 1994; Furger et al., 2001), as well as colon (Agrawal et al., 2002) and stomach (Ue et al., 1998).

The decreased osteopontin expression observed in the stomach tumor-bearing CA-AhR mice apparently contradicts the positive association with osteopontin expression and tumor progression observed by others. However, using osteopontin deficient mice in a chemically induced skin squamous cell carcinoma assay showed that these mice demonstrated accelerated tumor growth and progression, with a greater number of metastases (Crawford et al., 1998). Thus, osteopontin seem to play a complex role in tumor development that warrants further investigation.

Effects in the immune system

As discussed above in the section on "Expression and transcriptional activity of the CA-AhR", the initial intention was to direct expression of the CA-AhR to lymphatic cells via the use of expression vector containing the Eμ-enhancer from the immunoglobulin heavy chain locus. Effects on the immune system in the CA-AhR mice were mainly analyzed by FACS, focusing on the development and differentiation of B cells. These findings are presented in paper IV and briefly described and discussed below.

Effects observed on T cell populations

Experimental animals exposed to TCDD show atrophy of the thymus (Fletcher et al., 2001; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982). Moreover, prenatal exposure to TCDD results in altered distribution of thymic $CD4^+$ and $CD8^+$ populations (Gehrs et al., 1997). A decrease in thymus weight in CA-AhR is documented in paper I (Fig. 2E). FACS-analysis of thymocytes in adult CA-AhR animals did not show any altered distribution of single-positive $CD4^+$ or $CD8^+$ populations (data not shown). However, although not statistically significant, the $CD4^+$ population appeared to be decreased and the $CD8^+$ population appeared to be increased in newborn CA-AhR animals (data not shown). In contrast, peripheral T cells from the axillary and inguinal lymph nodes showed indications of an increase in population of $CD4^+$ and a decreased size of the $CD8^+$ T population (data not shown). No such differences were observed in spleen or mesenterial lymph nodes.

Increase in mature bone-marrow derived B cells

More consistent effects were observed in the B cell compartment of CA-AhR mice. Consistent with previous studies of TCDD-exposed mice (Thurmond and Gasiewicz, 2000), an increased fraction of mature B cells was found in the bone marrow of CA-AhR mice as assessed by FACS analysis of cells stained with several combinations of antibodies (Fig. 2, paper IV). Moreover, the fraction of mature B cells recognized by high levels of B220 staining and low level of staining for the monoclonal antibody 493, was also increased in the spleen of young CA-AhR mice (Fig. 3, paper IV). The reason for the observed enlargement of the mature B cell population is presently unknown.

Decreased peritoneal population of B1 cells

Effects caused by TCDD on the population of B cells found in the peritoneal cavity (B1 cells, see Introduction) has not been reported. However, in the CA-AhR mice a subpopulation of the peritoneal B1 cells (B1a), expressing the surface marker CD5, was substantially reduced in both the Y8 and A3 founder lines (Figs. 4A and 5A, paper IV). Moreover, according to the classical definition of B1a cells (Herzenberg et al., 1986), they express low levels of B220. The $CD5^{+}B220^{lo}$ population was reduced in the CA-AhR mice (Figs. 4C and 5B, paper IV), consistent with a selective loss of classical B1a cells. In contrast, the CD5-expressing B cells that remained in CA-AhR mice expressed substantially higher levels of B220 than those in wild-type mice (Fig. 4B, paper IV).

The lack or reduction of the $CD5^{+}$ B1 cell population in the peritoneal cavity has been reported in many different mouse models, most of which have deficiencies in molecules that modulate signaling from the antigen receptor (see the introductory section). Of certain interest are the mouse models overexpressing or deficient in the CD19 molecule which enhances signaling from the antigen receptor. Mice lacking CD19 show a similar decrease of the peritoneal $CD5^{+}B220^{lo}$ B cells, which in contrast are increased in mice overexpressing CD19 (Engel et al., 1995). Thus, CD19 appears to be an important regulator of $CD5^{+}$ B1 fate. Interestingly, decreased CD19 expression has been reported in cultured human B cells after TCDD exposure (Masten and Shiverick, 1995). However, we could not detect any difference in CD19 expression levels in either peripheral or bone marrow-derived B cells of adult mice and not in livers of newborn mice. A reduction of $CD5^{+}B220^{lo}$ B1 cells is also observed in chimeric mice lacking expression of Hif-1α in the immune system (Kojima et al., 2002). As described in the Introduction, both the Ah receptor and Hif-1α use Arnt as a dimerization partner to generate DNA-binding activity. It is therefore possible that functional inactivation of the Hif-1α signaling pathway by sequestration of a limiting pool of Arnt by the CA-AhR could explain the effect on the peritoneal B1 cells in CA-AhR mice.

Moreover, osteopontin (also called Eta-1 for Early T lymphocyte activation-1), which was reported to be decreased in several organs of the CA-AhR mice (paper III), is expressed in activated T cells and macrophages and plays an important role in cell-mediated (type-1) immunity (Ashkar et al., 2000; Denhardt et al., 2001). Mice lacking osteopontin show decreased resistance to viral (*Herpes simplex*) and bacterial infections (*Listeria*

monocytogenes) (Ashkar et al., 2000). In contrast, mice overexpressing osteopontin demonstrate an increased population of peritoneal B1 cells (Iizuka et al., 1998). Thus, it is tempting to speculate that the decrease in osteopontin mRNA expression levels observed in CA-AhR mice may contribute to the decreased population of B1 cells.

Conclusions

The aim of the present investigation was to characterize biological effects of CA-AhR in a transgenic mouse model. Some of the observations were in line with the expected outcome and others were more surprising. The results can be summarized as follows:

- The CA-AhR was expressed in all mouse tissues studied and was transcriptionally active in a ligand-independent manner as verified by induced expression of target genes.

- Effects previously observed in TCDD-exposed experimental animals were reproduced by the CA-AhR, such as thymus atrophy and increased liver weight.

- CA-AhR mice showed increased mortality that correlated with the development of cystic tumors in the glandular part of the stomach. These tumors penetrated the stomach wall, were constituted by well-differentiated cells, were actively proliferating and displayed intestinal and squamous metaplasia.

- Expression of osteopontin was decreased in the CA-AhR mice. This decrease seemed to depend on both the Ah receptor and Arnt since the rapid and transient decrease observed in wild-type hepatoma cells was not observed in hepatoma cell mutants deficient in these proteins.

- A significant reduction of the peritoneal population of CD5-expressing B1 cells was observed in CA-AhR mice, identifying this cell population as a novel target for the actions of the Ah receptor.

Taken together, these results show that the effects of the ligand-activated Ah receptor can be reproduced by the CA-AhR in the context of an intact animal. Moreover, the unexpected finding that the CA-AhR can induce tumors of the glandular stomach may provide a starting point for investigations of a possible physiological role for the Ah receptor in homeostatic control in the gastrointestinal tract. Consistent with such a role, several compounds derived from the diet (most notably, indolo-[3-2b]-carbinol) have been shown to bind and activate the Ah receptor. Primates and rodents exposed to Ah receptor ligands have showed stomach lesions reminiscent of the CA-AhR tumors. Proliferating tumor cells normally show some degree of dedifferentiation. In contrast, cells of the expanding and penetrating CA-AhR stomach tumors were highly differentiated. Compared to the vast literature on the control of proliferation and differentiation in the gut, not much is known about the molecular control mechanisms that govern this process in the stomach. A possible explanation could be an impairment in the control of either proliferation or apoptosis in CA-AhR mice. Obviously, these questions need to be further investigated. An alternative interpretation could be that the CA-AhR affects some central aspects of stomach cell differentiation, and experiments addressing this question by e.g. studying expression of established differentiation markers should be performed.

In addition to the main finding of stomach tumors, decreased expression of osteopontin and a reduced population of peritoneal B1 cells were other significant, but unexpected observations in CA-AhR mice. Although further analysis is needed for defining the mechanism behind these effects, some general speculations can be made. The role of osteopontin in tumor development seems to be complex and is not yet fully understood, and there may be a functional association between the decreased osteopontin expression and the stomach tumors in the CA-AhR mice. Since TCDD can disturb bone development (Jamsa et al., 2001; Singh et al., 2000), it will be interesting to analyze osteopontin expression in bones of CA-AhR mice. Although possibly coincidental, an interesting connection can be seen between the decreased population of peritoneal B1 cells and decreased expression of osteopontin. Mice that overexpress osteopontin show an increased population of these cells (Iizuka et al., 1998), and a decrease in osteopontin expression could conversely be related to a reduction of B1 cells. There is an increasing awareness of the importance of these cells in gastrointestinal immunity, and the so-called natural antibodies produced by the B1 cells are important in the defense against viruses, bacteria and parasites (Baumgarth et al., 1999; Baumgarth et al., 2000; Boes et al., 1998; Ochsenbein et al., 1999; Paciorkowski et al., 2000). Interestingly, decreased resistance to infectious agents is one of the most sensitive endpoints in experimental animals exposed to TCDD (Burleson et al., 1996; House et al., 1990), and one may speculate that a decreased B1 population may contribute to such an effect.

In summary, this thesis describes the first characterization of the CA-AhR mouse model, including the development of well-differentiated, penetrating stomach tumors, decreased expression levels of the osteopontin gene, and a reduced population of peritoneal B1 cells. These initial observations are only the starting point for the use of these animals in studies of biological processes regulated by the Ah receptor: e.g. gastrointestinal epithelial cell homeostasis which possibly defines an area of physiological receptor function.

Acknowledgements

These are probably the only pages that everyone reads, and minutes before leaving the thesis for print, I am so tired that it is difficult to find the words that really describe the feelings I have right now. There are so many people that over the years have contributed to this thesis in some way or the other that I would like to thank but probably will forget to mention and I apologize for that. However, there are some persons that have been essential for taking this project to the finish line and I especially would like to thank:

Annika, thanks for everything! For taking me on as a student when you were about to finish yourself; for keeping me as a student when we lost our professor; for always taking the necessary time for discussing all aspects of the project that we have encountered along the way; for eating pannkaka at Hjulet (det-var-bättre-förr) and for just being a great supervisor and friend.

Lorenz, my second supervisor, for accepting me in your lab where I have learned most (if not all) of the molecular biology I know together with high ambitions in scientific publishing, and also for sharing your fantastic quality of structuring and writing scientific texts and presentations which really helped out during the last weeks.

Sten Orrenius and Göran Pershagen, past and present Heads of IMM and Björn Öbrink, Björn Vennström and Urban Lendahl, past and present Heads of CMB for providing such stimulating and creative research environments.

Sara, for being such a great companion and friend in this project. Even though we have managed to avoid each other at work for one and a half year, we have definitely discussed most topics in the lab and at the weekly pannkaka, from the life as a Ph D student to the horrors at 30 meters depth in September and how to make the best havregrynsgröt.

Katta, for being a great friend and letting me ask all those stupid questions about cell cultures, western blot and other things that I should have learned a long time ago.

Camilla and Pekka at CMB for being great friends that really made the first years at CMB a blast. Lotta and Mattias at IMM for always being so happy and for sharing your laughs and good mood.

Gunilla for all the good work we did together and for your fantastic help taking care of all the hitopathological samples

Jacquie, for introducing me to the CA-AhR mice and the world of molecular biology in a very fun and enthusiastic way.

Anna, Carlos and Nikolai, my co-authors. Thanks for teaching me most everything I know about B cells and FACS and for nice lunches and dinners at Rålambshovsparken. Thanks for sharing your vast knowledge and enthusiasm for science in general, and the GI tract in particular. And thanks for being such a great SSH collaborator, we have many more things to do! (you can probably figure out yourself who I refer to).

All technical and administrative staff of the two groups: Gunilla, Kina, Ellu, Inga-Lill, Margareta, Margareta and Sirpa at IMM and Amina and Hannele at CMB. Thanks for taking care of all the work that most of us never see, but need so badly.

All the staff at the animal house during all these years, especially Pellina, Sylvia and Angelica

Anna Gräske och Carina Sehlstedt, for being important parts of the project and for good times during lunches and for Carina's strict discipline in the animal house.

Fredrik, Bill and now the last days Kristian, for all your computer help during these years.

My other co-authors: Sven P, Murray, Yo Kato, Petra and Andreas for contributing with your knowledge to this project.

Lisa for teaching me a technique that I unfortunately never used and Farzad for helping out with the *H. pylori* cultures.

Anders, Mats and Anna-Lena at the toxicology program; I have really learned a lot and had a good time working with you

Past and present members of Lorenz group in arbitrary order: Camilla, Pekka, Marika, Pilar, Pascal, Arunas, Helder, Jorge, Teresa, Magnus, Cecilia, Bill, Mia, Sallyann, Jacquie, Isabel, Yuichi, Kensaku, Keiji, Petra, Linda, Ingemar, Nina, Linda and Amina.

Past and present members of the Unit of Environmental risk assessment at IMM, Ulf, Helen, Lotta, Mattias, Gunilla, Fredrik, Sirpa, Maggan, Maggan, Ellu, Kina, Marie, Yvonne, Lasse, Sten, Inga-Lill, Katarina, Agneta, Christina, Salomon, Monica, Malin, Nicke, Natasha, Pi, David and probably several others for creating such a nice working atmosphere in the group!

The pathologists Sven Reiland, Ricardo Feinstein, Ivar Paulson and Björn Rozell as well as Ronny for all your valuable comments on the histopathology of these mice as well as the KI veterinarians Patricia and Anne for your help and interest during these years.

Other past and present friends at CMB: Sten, Jalle, Anethe, Paul, Virpi, Gittan, Tessi, Jeanette, Patrik, Johan, Helena, Carina, Monika, Mariorna, Krissi, and Gedas and at IMM: Martin, Kristian, Ilona, Kathrin, Tiziana, Therese, Lina, Lousie, Rickard, Ulf, Gerd, Marianne, Hannibal, Niklas, Karin, Janne, Åsa, Eva, Helene, Rolf, Ralf, Ulla, Johan, Annika, Bengt and Ian for all the good and fun times at work and off work.

All my other friends outside work, Jacob, Tinni, Staffan, Malin, Pär, Johanna, Fredrik, Kajsa, Sofia, Figge, Malena, Christian, Göran, Karin, Johan, Karna, Lasse, Alexandra, Ia, Johan, Eva, Macho, Johan, Erica and more…

My parents, Swedish and American, Charlotte's parents and my sister Nina for all your support during all these years and especially for taking all that time and the long-distance travel to help out in taking care of Linus during these last hectic weeks.

And finally, to them I love the most, for whom I really can't find the right words to express my feelings. Charlotte och Linus, jag älskar er och nu kommer jag hem!

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