Forschungszentrum Karlsruhe
Technik und Umwelt

Wissenschaftliche Berichte
FZKA 6201

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Januar 1999

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von der Fakultät für Bio- und Geowissenschaften
der Universität Karlsruhe (TH)
genehmigte Dissertation

Forschungszentrum Karlsruhe GmbH, Karlsruhe
1999

Als Manuskript gedruckt

Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe

Mitglied der Hermann von Helmholtz-Gemeinschaft
Deutscher Forschungszentren (HGF)

ISSN 0947-8620

Abstract

Dioxins, in particular TCDD, are potent mammalian toxins acting predominantly in the thymus and skin, in body weight regulation and in liver carcinogenesis. The Ah receptor (AhR) a ligand activated transcription factor belonging to the bHLH-PAS protein family mediates the toxicity of dioxins. Despite the extensive research conducted during the past 20 years, the mechanism by which AhR mediates the toxicity of dioxins is not understood. Known AhR regulated genes mostly code for xenobiotica metabolizing enzymes but the AhR target gene(s) which mediate toxicity are not known.

In this study 5L rat hepatoma cells were employed as a model system for dioxin toxicity in which TCDD severely delays cell cycle progression in the G1 phase by an AhR dependent mechanism. An AhR deficient variant subclone of 5L cells, the BP8 cells, are resistant to TCDD. These AhR-deficient cells were used for a mutational analysis of AhR overexpression to test the required properties of AhR to delay cell cycle progression. Both, the receptor's capacity for sequence specific DNA recognition and the presence of the transcriptional activation domain are necessary to induce the cell cycle delay. This suggests that AhR mediates the TCDD effects on cell cycle by *bona fide* induction of yet to be identified target genes. Such AhR target genes were searched following two approaches, e.g. based on the biochemical analysis of the cell cycle machinery and by a systematic search for AhR induced genes.

Evidence from biochemical analysis of the cell cycle machinery suggested that TCDD might induce cell cycle inhibitor(s). One of the inhibitory proteins, $p27^{Kip1}$, is induced by TCDD in 5L cells. Induction of $p27^{Kip1}$ occurs through the direct induction of Kip1 mRNA by AhR. AhR-dependent activation of Kip1-transcription is a novel mechanism of Kip1 induction which is distinct from the accumulation of Kip1 protein caused by posttranscriptional regulation in all the cases reported so far. Kip1 is the cause for TCDD-induced delay of 5L cell proliferation, since inhibition of Kip1 accumulation by expression of antisense RNA almost completely abolishes the effects of TCDD on the cell cycle. The induction of Kip1 could explain some of the toxic effects of TCDD *in vivo* which are associated with reduced proliferation, like the TCDD-induced atrophy of the thymus. Evidence for a role of Kip1 in thymus toxicity of TCDD has been generated in the group by the use of Kip1 deficient mice.

In a second approach, suppression subtractive hybridization was performed to identify TCDD-induced genes in 5L cells. Among the identified 34 differentially expressed genes, 22 were already known to be induced by TCDD.

In addition, there are 3 known genes whose expression was previously not known to be induced by TCDD and 8 novel sequences. A longer cDNA fragment corresponding to one of the clones was isolated and identified as part of the N-myristoyltransferase 2 (NMT-2) gene. TCDD is the first substance known to induce NMT-2 transcriptionally. NMT-2 is an enzyme catalysing myristoylation of many proteins, one of which is the signal transducing tyrosine kinase $p60^{Src}$. NMT-2 mRNA was also found to be increased in livers of mice treated with TCDD *in vivo*. Thus NMT-2 may play a role in liver carcinogenicity of dioxins, since increased myristoylation of proteins and, in particular, the Src family protein kinases is implicated in carcinogenesis.

In conclusion, the chosen approaches allowed the identification of several novel dioxin-induced target genes of the Ah receptor. One of the induced genes, the $p27^{Kip1}$ cell cycle inhibitor, was shown to be essentially required for dioxin toxicity in 5L cells. The complexity of changes in gene expression, as indicated by the independent induction of many and not obviously related genes, suggests that dioxins trigger multiple genetically defined response pathways rather than one `master gene´ responsible for all symptoms of dioxin toxicity.

Ah-(Dioxin) rezeptorabhängig regulierte Gene, die Dioxintoxizität vermitteln können: Induktion des $p27^{Kip1}$ Zellzyklusinhibitors und der N-Myristoyltransferase 2 durch Dioxine

Zusammenfassung

Dioxine und insbesondere TCDD sind potente Gifte für Säuger, die vor allem auf den Thymus, die Haut, das Körpergewicht und die Entstehung von Leberkrebs wirken. Der Ah-Rezeptor (AhR), ein Liganden-abhängiger Transkriptionsfaktor aus der bHLH-PAS Proteinfamilie, vermittelt die Toxizität von Dioxinen. Trotz immenser Forschungsanstrengungen während der vergangenen 20 Jahre ist der Mechanismus der Dioxintoxizität noch nicht geklärt. Bekannte AhR-abhängig regulierte Gene kodieren zumeist für Enzyme des Fremdstoffmetabolismus, aber AhR-regulierte Gene, die Dioxintoxizität vermitteln, sind nicht bekannt.

In dieser Studie wurden als Modellsystem der Dioxintoxizität 5L Rattenhepatomzellen analysiert, in denen TCDD über einen AhR-abhängigen Mechanismus die Progression des Zellzyklus durch die G1-Phase erheblich verzögert. Ein AhR-defizienter Subklon der 5L-Zellen, die BP8-Zellen, ist resistent gegenüber TCDD. In diesen AhR-defizienten Zellen wurde der AhR ektopisch exprimiert und eine Mutationsanalyse durchgeführt, um die für den Zellzyklusarrest erforderlichen Eigenschaften des AhR zu testen. Sowohl die Fähigkeit an spezifische DNA-Erkenungssequenzen zu binden als auch die transaktivierende Funktion des AhR sind notwendige Voraussetzungen für die Hemmung der Zellzyklusprogression. Diese Beobachtung deutet darauf hin, daß der AhR die TCDD-Effekte auf den Zellzyklus durch bona fide Induktion von im weiteren zu identifizierenden 'Toxizitätsgenen' vermittelt.

Hinweise aus der biochemischen Analyse der Zellzyklusmaschinerie wiesen darauf hin, daß TCDD einen oder mehrere Zellzyklusinhibitor(en) induzieren könnte. Eines der Inhibitorproteine, $p27^{Kip1}$, wird durch TCDD in 5L-Zellen induziert. Diese Induktion beruht auf der direkten Induktion der Kip1 mRNA durch den AhR. AhR-abhängige Aktivierung von Kip1-Transkription stellt einen neuen Mechanismus der Kip1-Induktion dar, der sich von allen Formen der bisher beschriebenen posttranskriptionellen Regulation unterscheidet. Kip1 ist die Ursache für die Verzögerung des Zellzyklus durch TCDD, weil die

Verhinderung der Kip1-Akkumulation durch Expression einer Kip1 Gegensinn-RNA die Dioxineffekte auf den Zellzyklus nahezu vollständig unterdrückt. Die Induktion von Kip1 könnte auch einige der toxischen Dioxinwirkungen in vivo erklären, die, wie zum Beispiel die TCDD-induzierte Atrophie des Thymus, mit reduzierter Zellproliferation assoziiert sind. Die Analyse Kip1 defizienter Mäuse in der Arbeitsgruppe zeigt tatsächlich Evidenz für die Rolle von Kip1 bei der Thymustoxizität von TCDD.

In einem zweiten Ansatz wurde die Methode der 'Suppression subtractive hybridisation' eingesetzt. um TCDD-induzierte Gene in 5L-Zellen zu identifizieren. Unter den 34 isolierten differentiell exprimierten Genfragmenten fanden sich 22 Gene, deren Induktion durch Dioxine schon beschrieben wurde. Zusätzlich fanden sich 3 bekannte Gene, deren Induktion durch TCDD bisher nicht bekannt war, sowie 8 unbekannte Genfragmente. Die Isolation eines längeren cDNA-Fragmentes zu einem dieser unbekannten Fragmente führte zu dessen Identifizierung als Teil des N-myristoyltransferase-2 Gens (NMT-2). TCDD ist der erste beschriebene transkriptionelle Induktor der NMT-2-Expression. Das Enzym NMT-2 katalysiert die Myristoylierung einer Vielzahl von Proteinen, von denen eines die signalübertragende $p60^{Src}$ Protein-Tyrosinkinase ist. Erhöhte Spiegel der NMT-2 mRNA wurden auch in der Leber TCDD-behandelter Mäuse gefunden. Damit könnte NMT-2 eine Rolle bei der Leberkanzerogenität von Dioxinen spielen, denn der Proteinmyristoylierung und insbesondere die Myristoylierung von Proteinen der Src-Familie wird eine Bedeutung bei der Kanzerogenese zugemessen.

Zusammenfassend erlaubten die gewählten Ansätze die Identifizierung mehrerer neuer Dioxin-induzierter Zielgene des AhR. Für eines der induzierten Gene, Kip1, konnte eine essentielle Rolle für die Dioxintoxizität in 5L-Zellen nachgewiesen werden. Die Komplexität der Veränderungen in der Genexpression, die sich in der Induktion vieler offensichtlich von einander unabhängiger Gene ausdrückt, deutet darauf hin, daß Dioxine mehrere genetisch definierte Programme triggern und kaum von einem 'Schlüsselgen' für alle Symptome der Dioxintoxizität auszugehen ist.

Table of contents

Abbreviations

A	Adenosine
AHH	Aryl hydrocarbon hydroxylase
Ahr	Aryl hydrocarbon responsiveness
AhR	Aryl hydrocarbon receptor
ANF	α-Naphthoflavone
APS	Ammonium persulfate
AR	Androgen receptor
Arnt	Ah receptor nuclear translocator
bp	base pair
bHLH	basic Helix Loop Helix
BrdU	Bromodeoxyuridine
BTG1	B-cell translocation gene
C	Cytidine
°C	Degrees celsius
CAK	CDK-activating kinase
cDNA	complementary DNA
CDK	Cyclin dependent kinase
CKI	CDK inhibitory protein
cm	centimeter
CY	Cytochrome
CYP	Cytochrome protein
DMEM	Dulbecco's modified eagles medium
DMF	N,N'-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin responsive element
ds	double strand
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamine-N,N-tetracetate
e.g.	example given
EGF	Epidermal growth factor
et al.	and others (Lat. *et ali*)
FACs	Fluorescence activated cell sorter
FBS	Foetal bovine serum
f.c.	final concentration

g	gram
G	Guanosine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GST-YA	Glutathion-S-transferase Ya
^3H	Tritium
h, hr	hour
HSB	High salt buffer
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
kb	kilobase (1kb=1000bp)
l	liter
LOT1	Lost on transformation-gene
kDa	kilodalton (10^3 daltons)
KiP1	Kinase inhibitory protein
m	milli
M	molar
mA	milliampere
Mab	monoclonal antibody
MAPK	Mitogen activated protein kinase
mg	milligram (10^{-3} gram)
min	minute
ml	milliliter (10^{-3} l)
mRNA	messenger RNA
µ	micro-
µg	microgram 10^{-6} gram)
µl	microliter (10^{-6} l)
mM	millimolar (10^{-3} molar)
µM	micromolar (10^{-6} molar)
n	nano-
ng	nanogram (10^{-9} gram)
nm	nano meter (10^{-9} meter)
NMT	N-Myristoyltransferase
OD	Optical Density
o/n	over night
p	pico-
PAGE	Polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbons

PAS	Per Arnt Sim- homology domain
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
PEG	polyethylene glycol
pg	picogram (10^{-12} gram)
PK-A	Protein kinase A
RACE	Rapid amplification of cDNA ends
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse Transcription PCR
RT	room temperature
SAPK	Stress activating protein kinase
SDS	Sodium-Lauryl-Sulfate (sodium dodecyl sulfate)
sec	second
ss	single strand
SSH	Suppression Subtractive Hybridisation
T	Thymidine
TAD	Transcriptional activation domain
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	N,N,N',N'-tetramethylethylenediamine
TEQ	Toxicity equivalent
TGFβ	Transforming growth factor β
TRIS	Tris-(hydroxymethyl)-aminomethane
U	unit(s)
UDP	Uridinediphosphate
UV	ultraviolet
V	Volt
W	Watt
XRE	Xenobiotica responsive element

1. Introduction

The term 'Dioxins' is commonly used to describe a family of extremely toxic compounds, e.g. the polyhalogenated dibenzo-p-dioxins. All eight hydrogens in the dibenzo-p-dioxin molecule can be substituted by halogen atoms in various combinations. Only considering all possible chlorinated dioxins allows the formation of 75 chemically distinct congeners. Dioxins have never been produced by intention except for scientific purposes. Rather, dioxins are formed as by-products in many organic chemical reactions or combustion processes in the presence of halogens. Major sources of dioxins are the production of chlorinated phenols or phenoxy herbicides and pesticides, waste incineration, metal smelting, combustion of leaded fuels and chlorine bleaching of paper and pulp. Many of these processes release dioxins ubiquitously into the environment. As a consequence, some of the dioxin-generating processes have been dramatically improved or have been abandoned since the problem of dioxin contamination became apparent. One of the dioxins, the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has gained special interest and serves the role of a model compound because of its high toxicity. Together with dioxins usually two related classes of halogenated aromatic hydrocarbons, the halogenated dibenzofuranes and biphenyls, are generated which can have similar effects on the biosphere. Hence a broader definition of the term dioxins usually includes the dibenzofurane-derived compounds (figure 1).

Dioxins and TCDD in particular have gained a high degree of public attention because they are toxic to human and animals at very low doses. TCDD was in the focus of public interest when in 1976 at the Seveso (Italy) chemical plant large quantities of TCDD and other dioxins were released into the environment and severely intoxicated the local population with chloracne being the acute and most prominent symptom. Other large-scale intoxication of humans occurred through contaminated rice oil in Yusheng (Taiwan), at chemical plants in Bophal (India) and Hamburg (Germany). A heavy public, legal, and scientific debate was fuelled by the intoxication of American Vietnam soldiers by dioxins which had been present as a contaminant in 'Agent Orange' which was used for defoliation and crop destruction. However, it is not the few cases of heavy intoxication, but rather the ubiquitous presence in the environment, the accumulation and persistence in the food chain leading to dioxin exposure of humans which have kept dioxins in the public discussion since. Despite of this general interest and some success in understanding biological activities of

dioxins, the mechanisms how dioxins are toxic to mammals are not yet understood.

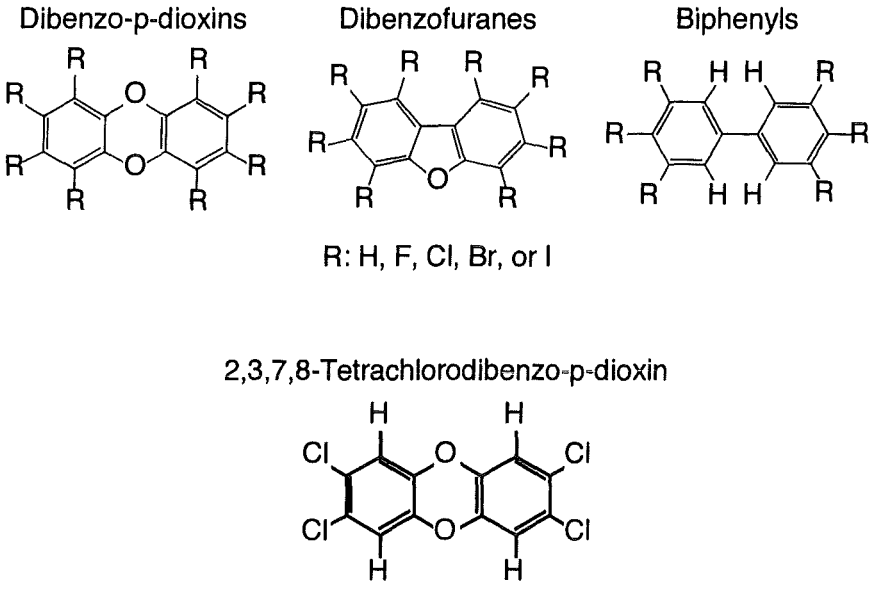

Figure 1. Chemical structures of 'dioxins' and related biphenyls. The basic structures of dibenzo-p-dioxins, dibenzofuranes and the biphenyls are shown. Compounds with 'dioxin'-like biological activities contain halogen atoms, mostly chlorine, instead of hydrogen in numerous combinations at some of the positions indicated by R. The most potent congener and model compound 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (lower panel) contains chlorine atoms in the 4 lateral positions.

1.1 Biological effects of dioxins in animals

Laboratory animals including primates have been extensively used to study dioxin toxicity. The prominent effect of dioxins in laboratory animals is a wasting syndrome which is characterized by progressive loss in body weight which ultimately results in death (Huff et al.,1994 and Christian et al.,1986). The sensitivity to TCDD varies among different species. Guinea pigs have been shown to be highly susceptible to the lethal effects of TCDD. The hamster is approximately 5000 times less sensitive, e.g. the LD_{50} for acute TCDD poisoning is 1 µg/kg body weight in the guinea pig and 5000 µg/kg in the hamster. Rats and mice show intermediate sensitivity (Poland et al., 1982). Many other symptoms of acute or subacute toxicity occur at much lower doses. The immune system for example is a sensitive target of TCDD toxicity. The effects include thymic atrophy (Fine et al., 1990 and Silverstone et al., 1994a), degenerative changes in bone marrow (Silverstone et al., 1994b; Frazier et al., 1994 and Staples et al., 1998),

and alterations in the immune response affecting both humoral and cell-mediated immunity (Fine et al., 1990 and Silkworth et al., 1986). The generation of sperm cells is affected resulting in a reduced sperm count. Toxic effects on the skin are found in nude mice (Knutson and Poland, 1982). Dioxins are also toxic to the developing embryo resulting in increased rates of fetal mortality or malformations like cleft palate and skeletal abnormalities (Peterson et al., 1993 and Couture et al., 1990).

TCDD is a carcinogen in several rodent species, including the hamster. In rats TCDD induced neoplasms in lung, oral and nasal cavities and liver (Kociba et al., 1978). In mice it caused neoplasms in liver, subcutaneous tissue, lung and lymphopoietic system (Toth et al., 1979) and in hamsters it produced squamous cell carcinomas of the facial skin (Rao et al., 1988). TCDD is carcinogenic at doses as low as 1 ng/kg body weight per day which is far below the lethal dose (Huff et al., 1994). TCDD is, however, not mutagenic and this is consistent with the findings that it does not directly bind to DNA and form DNA adducts (Turteltaub et al., 1990). Thus, by the simple model of tumor initiation and promotion/ progression, TCCD would act as tumor promoter rather than an initiator. TCDD is considered a potent carcinogen because it is carcinogenic at quite low doses compared to other tumor promoters.

1.2 Effects of dioxins in humans

Dioxin exposure and toxicity in humans is relevant in three types of populations, the acutely poisoned people in accidents like those at Seveso, people exposed occupationally during their work life, and the general population which is exposed to low levels of dioxins through environment and food chain.

The predominant effect observed in humans exposed to relatively large amounts of TCDD is chloracne. Chloracne is a severe skin disease characterized by follicular hyperkeratosis occurring with or without cysts and pustules. Carcinogenicity of dioxins in rodents raised the concern that dioxins may also be human carcinogens and this proposition is supported by the follow up study of the dioxin-poisoned population of Seveso. With a latency of up to now 20 years increased rates of soft tissue sarcomas, lymphoid and myeloid neoplasms as well as stomach and rectal cancer are found clearly supporting the view that dioxins are human carcinogens (Bertazzi et al., 1993, 1997 and Pesatori et al.,

1993). The strength of such studies lies in good knowledge about the initial intoxication but the draw back from a statistician's point of view is the relatively low numbers of individuals.

Epidemiological data from occupationally exposed workers have established an association between exposure to TCDD and several human cancers, especially digestive and respiratory cancers (Flesch-Janys et al., 1995, Kogevinas et al., 1997; Manz et al., 1991; Ott and Zober, 1996; Smith and Bates, 1992 and Smith et al., 1992). A number of diseases other than cancer have also been reported which include infectious and parasitic diseases, skin and liver diseases, respiratory tract infections and mental disorders (Zober et al., 1994). The difficulty in these studies is that occupational exposure probably was not only to dioxins but also other non-dioxin hazardous compounds. Although best efforts have been taken to compensate for such potential influences for example by work history exploration and direct measurements of dioxin body burdens, such studies are more difficult to interpret than the single exposures in the few accidents.

The major concern in the public discussion is the low level of dioxin exposure through the environment. It is estimated that exposure levels to 2,3,7,8-TCDD in industrialised nations are approximately 20 to 40 pg per day. Effects induced by 2,3,7,8-TCDD are also elicited by other dioxins (Rodriguez-Pichardo et al., 1991) and usually average exposure is to a complex mixture of several dioxins. Different dioxins differ dramatically with respect to their potency, e.g. required doses *in vivo* or concentrations in cultured cells, compared to 2,3,7,8-TCDD. The biological system responds to the cumulative exposure to these chemicals rather than to any single compound. To account for such cumulative effects, each toxic congener has been given an equivalence factor which allows to transform the actual dose or concentration of any congener to the amount of 2,3,7,8-TCDD which would have the same biological activity, the so-called 'toxicity equivalent' (TEQ). The rational for such a comparison is based on the knowledge discussed below on a common primary intracellular target for TCDD-like dioxins (Eadon et al., 1986). Taking into consideration exposure to all relevant dioxins including polychlorinated biphenyls, dibenzofuranes, and other dibenzo-p-dioxins results for adults an average uptake of 200 to 400 pg TEQ per day. The most important dioxin uptake occurs with the food. Due to their lipophilic and chemically inert properties, dioxins are found particularly in products which are made from late stages in the food chain like animal products or milk fats. The

biological half life of TCDD in humans is about 7 years and thus the daily uptake accumulates to substantial levels.

The accumulated body burdens in humans are within one order of magnitude close to those which show signs of toxicity in rodents. There is, however, no indication, that the average burdens of dioxins cause any diseases in humans. This may be because humans are not very sensitive or because of the inability to detect adverse effects of dioxins with the existing scientific tools. Reasons for these difficulties could be that doses after all are low and only few particularly sensitive people are affected. Another reason could be based on the limited power of epidemiological studies because there is no non dioxin-exposed control population which a dioxin-exposed population can be compared to. Comparison of low versus high level exposed subpopulations based on their dioxin levels in the blood lipids may be an approximation of limited power.

Understanding the mechanisms by which dioxins are toxic in rodents could help to extrapolate to humans and could particularly help to predict at which doses or body burdens dioxin toxicity can be expected. The realisation that there is an intracellular receptor binding dioxins and cloning of that receptor have been important, but by far not sufficient, steps towards understanding the mechanism(s) of dioxin toxicity.

1.3 Evidence for a dioxin binding, intracellular receptor

In rodents or in cultured cells polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene or methylcholantherene induce the **a**ryl **h**ydrocarbon **h**ydroxylase activity (AHH), which is associated with a number of individual cytochrome P450-dependent monooxygenases, e.g. the CYP450 1A and 1B families. The inducibility of AHH activity varied among mouse strains, with C57BL/6 mice being highly responsive to PAHs, where as DBA/2 mice were only poorly responsive. The F1 offsprings after crossing these two strains showed high responsiveness to PAHs. Backcrosses to the parental strain showed that the high AHH inducibility is inherited as an autosomal dominant trait and resulted in the postulation of a gene locus which was named as *Ahr-* locus (*Ahr* for Aryl hydrocarbon responsiveness).

Experimental evidence that the *Ahr*-locus encodes a protein for binding aromatic hydrocarbons came from studies using TCDD which at very low doses induced AHH in the 'responsive' but not in the 'non-responsive' mouse strains. Ligand-binding studies with ^{3}H-TCDD, analogous to those performed with steroid hormones, demonstrated the presence of high affinity, and saturable binding sites in cytosolic extracts from 'responsive' mice but not from the 'non-responsive' mice. The presence of high affinity binding sites segregated with the 'responsive' phenotype in the crosses between 'responsive' and 'nonresponsive' strains. Binding of TCDD could be competed by other AHH-inducing aromatic hydrocarbons. This indicated the presence of a receptor binding TCDD and aryl hydrocarbons which was named the Ah receptor (AhR) (Swanson and Bradfield, 1993).

Due to its low abundance, the receptor has resisted purification for almost 20 years. The development of a photoaffinity ligand carrying a radioactive iodine atom had finally allowed purification and N-terminal amino acid sequencing of the receptor (Perdew and Poland, 1988). The degenerate cDNA sequence deduced from the peptide sequence allowed cloning of the AhR's cDNA from recombinant cDNA and genomic libraries (Burbach et al., 1992 and Ema et al., 1992).

1.4 AhR is a ligand activated transcription factor

Much of the knowledge on AhR function was gathered before the AhR gene was cloned by biochemical and cell biological methods mostly using the inducibility of AHH activity in cultured Hepa1 murine hepatoma cells as the experimental system. In these cells the unliganded receptor is found exclusively in the cytoplasm bound to heat shock protein 90 (HSP90) (Denis et al.,1988; Okey et al., 1994 and Perdew, 1988). Upon ligand binding (e.g. TCDD), the Hsp90 gets dissociated and the ligand-bound receptor translocates into the nucleus (Okey et al., 1994). AHH activity in these cells is mostly constituted by the cytochrome P450 1A1 enzyme. Cytochrome P450 1A1 is induced by AhR ligands on the level of increased gene expression and hence the gene encoding this enzyme, e.g. the *CYP450 1A1* gene, has been studied as a model case how the nuclear AhR can regulate gene expression.

The cloning and deletion analysis of the 5' regulatory region of the *CYP450 1A1* gene has revealed the existence of several short sequence elements, each of which can mediate enhanced gene expression upon activation of the AhR. These elements are functional in the context of the *CYP450 1A1* promoter or when placed in front of other initially not AhR responsive promoters (Hankinson, 1995; Nebert et al., 1993 and Whitlock, 1990). Thus these short DNA sequences fulfil the criteria of a true transcriptional enhancer element and have been called dioxin responsive elements (DREs). Apart from dioxins also many other non physiologic compounds like non-halogenated aromatic hydrocarbons or aromatic amines (Cikryt et al., 1990 and 1992) induce *CYP450 1A1* through these elements and therefore they are also called XREs for xenobiotica responsive elements..

The nuclear translocation of activated AhR and the presence of specific DREs in the 5' regulatory region of an AhR activated gene strongly suggested that AhR acts as a ligand-activated transcription factor much like the steroid hormone receptors. This mode of action would require specific binding through one domain of AhR to short recognition sequences in the regulated target gene promoter, e.g. the DRE/XRE, and activation of transcription through a second so-called transactivation domain. The first requirement, the sequence-specific binding to the XRE, has been shown to be matched by the presence of a XRE-binding activity in extracts from TCDD treated Hepa1 cells in a gel mobility shift analysis (Carlstedt-Duke et al., 1981; Denison et al., 1989 and Fujisawa-Sehara et al., 1988). The observations that this DNA-binding activity was not present in cells which had not been treated with TCDD or in TCDD-treated mutant Hepa1 cells which lacked a functional AhR proved that the DNA-binding activity was specifically associated with AhR (Hapgood et al., 1989). The most simple explanation of this observation was that AhR itself is a DNA-binding molecule. The experimental evidence for this proposition was generated much later after cloning the AhR cDNA and generation of AhR directed antibodies (Poland et al., 1991).

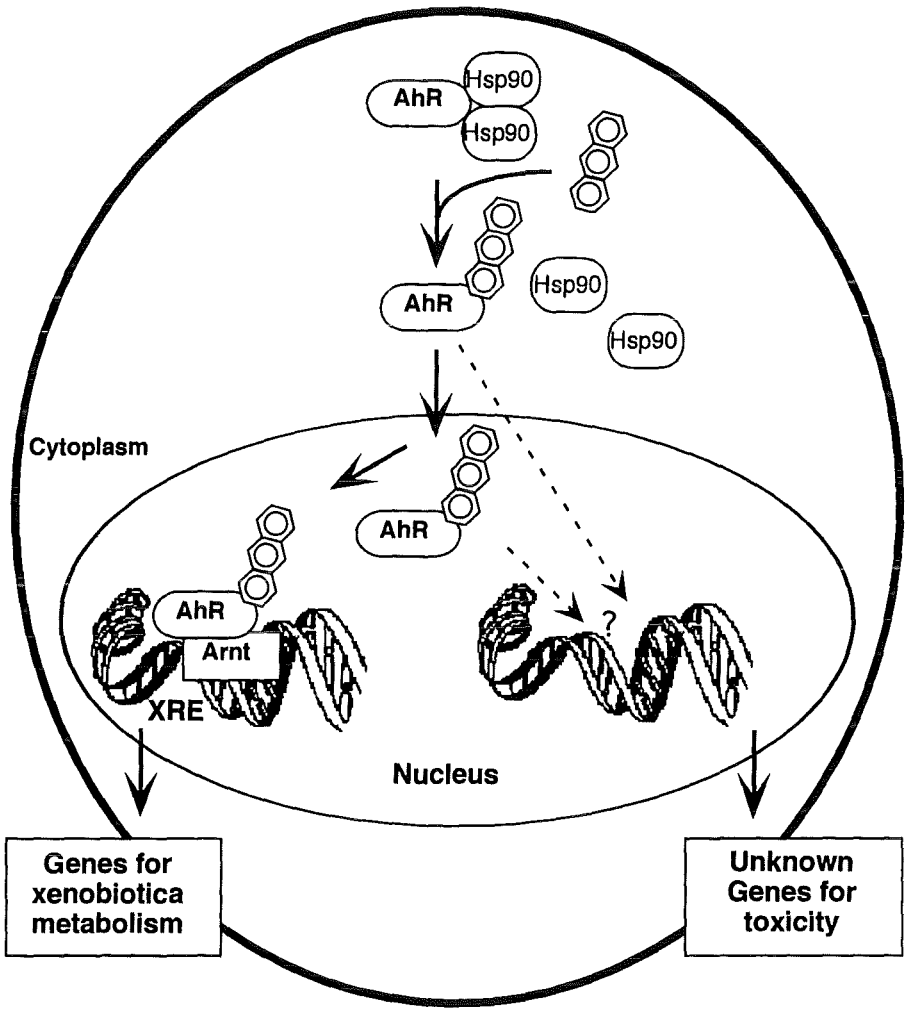

Figure 2. Outline of the function of the Ah receptor (AhR) as a ligand-activated transcription factor. AhR is found in cytoplasm bound to heat shock protein 90 (Hsp90) in the absence of a ligand. Ligand binding results in release of Hsp90 and nuclear translocation of AhR. The receptor dimerises with Arnt. The heterodimeric complex binds to specific sites, so-called xenobiotica responsive elements (XREs), in the promoters of genes coding for xenobiotic metabolising enzymes and activates their transcription. AhR regulated genes mediating dioxin toxicity are not known.

The Hepa1 cell line and the derived mutant cell lines which lacked AHH inducibility were also used to generate the initial evidence that several genetic loci contribute to AHH inducibility by the AhR system (Legraverend et al., 1982). The AhR-deficient clones described above only constituted one complementation group which appeared by the lack of detectable ^{3}H-TCDD binding to AhR. Another complementation group appeared by the presence of ligand binding AhR which however did not properly translocate to the nucleus. Complementation cloning in these deficient cells allowed identification of the gene responsible for this defect and the protein encoded by that gene was called the as **A**h **r**eceptor **n**uclear **t**ranslocator (Arnt) (Hoffman et al.,1991). Later studies have suggested that Arnt is not directly involved in translocating AhR into the nucleus, because AhR can be found in the nucleus after ligand treatment in Arnt deficient Hepa cells (Pollenz et al., 1994). Using antibodies generated against Arnt, it was shown that Arnt was a part of the complex binding to the XRE together with AhR (Reyes et al., 1992) (figure 2). The consensus sequence of the XRE was identified as 5' TC/TGCGTG 3'. AhR binds to the 5' (TC/TGC) and Arnt to the 3' (GTG) half sites of the XRE (Swanson et al.,1995).

Besides *CYP450 1A1*, genes for other xenobiotic metabolizing enzymes are regulated through XREs, including *CYP450 1A2, CYP450 1B1* and non-P450 enzymes like glutathione-S-transferase Ya, UDP-glucuronosyltransferase, NAD(P)H:quinone oxidoreductase and aldehyde dehydrogenase.

1.5 AhR and Arnt are members of the basic-helix-loop-helix-PAS family of transcription factors

Cloning of the AhR and Arnt cDNAs revealed that they are distinct from steroid hormone receptors. Amino acid sequence alignments showed a striking resemblance in overall structure of AhR and Arnt (Burbach et al., 1992; Ema et al., 1992; Hoffman et al., 1991 and Itoh and Kamataki 1993). They contain basic-helix-loop-helix (bHLH) motifs near their amino termini. Such motifs are found in other transcription factors like Myc, Max and MyoD that bind specific DNA sequences as homodimers and heterodimres, where they function in both protein dimerization and DNA binding (Davis et al., 1990 and Murre et al., 1989). A feature of many bHLH proteins is the presence of a secondary dimerization surface adjacent to the HLH domain. An example of such a secondary

dimerization domain is the leucine zipper, and bHLH proteins containing this motif are called bHLH-ZIP proteins (Kadesch, 1993).

The AhR and Arnt contain a segment of homology adjacent to the bHLH motif, which is also found in two regulatory proteins of Drosophila, Per and Sim (Huang et al., 1993 and Nambu et al., 1991). The homologous domain present in all four proteins has been termed the PAS domain, for **P**er-**A**hR/Arnt-**S**im homology domain. The PAS domain contains two copies of an approximately 50-amino acid degenerate direct repeat, referred to as the PAS A and PAS B repeats (Citri et al., 1987; Crews et al., 1988 and Nambu et al., 1990). Both AhR and Arnt contain glutamine-rich regions near the carboxy termini. Glutamine rich regions are also found in other transcription factors such as SP1, and function as transcriptional activation domains (Jain et al., 1994 and Whitelaw et al., 1994).

The AhR is unique among bHLH proteins as its activity is dependent on the binding of a ligand. Hypoxia inducible factor 1α (HIF-1α) is a new member of the PAS family and regulates transcription of genes in response to hypoxia as a heterodimeric complex with Arnt (Wang and Semenza, 1995). Per is an unusual member of the PAS family without a bHLH domain (Huang et al., 1993).

1.6 Functional domains of AhR and Arnt

Functional domains of AhR and Arnt were investigated by deletion analysis by several groups. The basic region of both AhR and Arnt are required for DNA binding (Bacsi and Hankinson 1996 and Swanson and Yang 1996). The PAS domain is necessary and sufficient for dimerization (Lindebro et al., 1995 and Weiß et al.,1996) of both proteins. The bHLH domain is shown to stabilize the heterodimers. Ligand binding of AhR is localized to a region encompassing the PAS B repeat in AhR (Fukunaga et al., 1995 and Whitelaw et al., 1993). The PAS domain of AhR also harbours the contact region for association with HSP90. This binding is necessary for the ability of AhR to assume and/or maintain a ligand binding confirmation since the receptor loses its affinity for its ligand if HSP90-receptor complex is disrupted (Pongratz et al., 1992 and Whitelaw et al., 1995). C-terminal glutamine-rich regions of both AhR and Arnt have potent transcriptional activation domains (TADs) (Hui Li et al., 1994; Jain et al., 1994 and Weiß et al., 1996) which function on heterologous DNA binding domains. Which TAD prevails in gene activation in a given context seem to depend on the

assay system (Fukunaga et al., 1995, Whitelaw et al., 1994, and results presented in this thesis).

1.7 AhR mediates the toxicity of TCDD

AhR mediates the toxicity and carcinogenicity of TCDD and its structural analogues based on several observations. Firstly, structure-activity relationships demonstrate that within a particular series of halogenated aromatic hydrocarbons (for example, polychlorinated dioxins and polychlorinated biphenyls), the toxicity of a compound is correlated with the affinity with which it binds to AhR. Secondly, mice strains differ in their susceptibility to the toxic effects of TCDD. The AhR from the nonresponsive strains of mice such as DBA/2 binds TCDD with an affinity that is about tenfold lower than the receptor from the responsive strains such as C57BL/6 mice. This difference in affinity parallels the difference between the strains in their sensitivity to the biochemical and toxic effects of TCDD (Swanson and Bradfield, 1993). Lastly, studies of AhR knockout mice indicated that mice lacking the receptor are resistant to TCDD-induced toxic effects (Fernandez-Salguero et al., 1996).

Despite the extensive research conducted during the past several years, the mechanism by which AhR mediates the effects of dioxins is not known. Much of what we know about AhR function comes from the studies of *CYP450s* and other xenobiotic metabolizing enzymes. Function of these genes is not likely to explain dioxin toxicity. However, AhR regulated genes which mediate dioxin toxicity are unknown. No link has been demonstrated between the induction of xenobiotic metabolizing enzymes and any toxic end points of dioxins.

A number of groups have attempted to identify changes in gene expression following TCDD exposure by 'educated guess' and 'differential screening' approaches. The changes in gene expression which could lead to alteration in differentiation and proliferation of cells upon TCDD treatment were analysed. Members of the AP-1 family of proto oncogenes like C-*jun*, c-*fos* and *jun*-D were shown to be induced by TCDD in mouse and rat hepatoma cells (Hoffer et al.,1996; Nebert et al., 1993; Puga et al., 1992 and Carsten Weiß, personal communication). However, the mode of induction of these genes is not clear and data in the literature are not conclusive.

Using differential hybridization, two TCDD-responsive genes, plasminogen activator inhibitor 2 and interleukin 1β were isolated from human keratinocytes (Sutter et al., 1991). Though it was shown that both the genes were induced at the transcription level, it could not be ruled out that the induction was a consequence of differentiation of the used keratinocytes. Neither of these genes is induced in other cell systems for example in 5L cells (Own unpublished observations and W. F. Greenlee, personal communication) or in livers of TCDD-treated rats (Fox et al., 1993 and Vanden Heuvel et al., 1994). The differential display technique was employed to isolate a novel TCDD-induced gene from rat liver with sequence homology to a cytokine/growth factor/prolactin receptor superfamily (Selmin et al., 1996). Very recently, TCDD also was shown to induce ecto-ATPase in mouse hepatoma cells (Gao et al., 1998).

Induction of these genes provide evidence that TCDD alters different sets of genes in different cells/organs. But there are no studies published linking the induction of these genes and TCDD toxicity. Thus, there is considerable interest in identifying more AhR regulated genes and determining which of these genes are responsible for the manifestations of TCDD toxicity.

1.8 Model system for TCDD toxicity

Most of the toxic effects of TCDD are associated with changes in differentiation and proliferation of cells. I chose a model system which allows to study the effects of TCDD on proliferation.

One of the few toxic actions of TCDD in continuously growing cell culture is a severe delay in G1-S progression of 5L rat hepatoma cells (Göttlicher and Wiebel 1991). This effect of TCDD on cell proliferation is not found in BP8 cells, the variant clones of 5L cells which lack AhR, suggesting that AhR is mediating TCDD effects on cell cycle progression (Göttlicher et al., 1990). Stable expression of AhR in the receptor deficient BP8 cells, reconstitutes the TCDD-induced cell cycle delay (Weiß et al., 1996). Thus AhR plays a causal role in mediating the TCDD-induced cell cycle delay in 5L cells. However, it is not known which genes are regulated and how they are regulated. It is also not known whether changes in gene expression are the primary cause at all for the AhR-mediated cell cycle delay.

AhR is known to mediate diverse effects of TCDD apart from transcriptional activation of XRE regulated genes. The AhR was shown to interfere with growth factor signalling (Astroff et al., 1990; Hudson et al., 1985 and Lin et al., 1991), to bind to cell cycle regulatory molecules like the retinoblastoma protein and cyclin-CDK complexes (Ge and Elferink, 1998 and Greenlee, personal communication) and also to increase tyrosine kinase activity of $p60^{c\text{-}Src}$ (Enan and Matsamura, 1995). From steroid hormone receptor studies there is evidence for different modes of nuclear receptor action independent of binding to DNA and direct transcriptional activation of genes. The steroid receptors can transrepress genes and also induce signal transduction cascades (Göttlicher et al., 1998; Jonat et al., 1990; Migliaccio et al., 1996; Peterziel, 1998 and Schuele and Evans, 1991). Thus it appears possible that AhR can also have fuctions independent of direct activation of genes. So, it is important to determine whether all functions of AhR as an activator of transcription are also required for mediating TCDD-induced cell cycle delay.

1.9 Regulation of the cell cycle

Living cells repeatedly grow and divide in a cyclic fashion and the period from one division to the next is called the cell cycle. The duration of the cell cycle in animal cells is usually 18-24 hours and is divided into 4 phases namely G1, S, G2 and M- phases (for **G**ap, **S**ynthesis and **M**itosis). After mitosis, cells are released into a condition as G1 phase. This is the usually longest phase in the cell cycle and its duration varies in different cells. Commitment to chromosome replication occurs in G1 phase. The initiation of DNA replication marks the transition from G1 phase to S phase. During S phase, the genome is replicated and the total content of DNA increases from the diploid value of 2n to the fully replicated value of 4n. The period from the end of S phase until mitosis is called G2 phase. During this period, the cell has two complete diploid sets of chromosomes. Mitosis of a cell generates two identical daughter cells, each bearing a diploid set of chromosomes. This period corresponding to the actual division is called the M phase (Heichman and Roberts, 1994). After division, the cells start with G1 phase again. Since the effect of TCDD is in the G1 to S phase progression, only the events of this part of the cell cycle are presented in more detail here.

1.9.1 G1 to S phase progression

The transition of cells through G1 to S phase of the cell cycle involves major check points which allow modulation of proliferation by signals from inside or outside the cell. The commitment of cells to enter the S phase occurs at the restriction (R) point in mid/late G1 phase, after which growth factors are no longer required for cells to complete division (Pardee, 1989).

Phosphorylation of the retinoblastoma tumor suppressor gene product (pRb) coincides with the R point of transition and is also likely to play an essential role in commitment to replication. In its hypophosphorylated state pRb binds to and negatively regulates transcription factors such as E2F, whose functions are important for G1 to S phase progression (Bartek et al., 1996 and Weinberg, 1995). Phosphorylation of pRb during middle to late G1 phase reverses the growth suppressive effects of pRb by releasing E2F, there by enabling it to activate genes required for DNA replication. Cyclin D and E- associated kinase activity is required for phosphorylation of pRb in G1 phase and progression of cells from G1 to S phase.

Three D-type cyclins (D1, D2 and D3) are expressed early in G1 phase and complex with either CDK4 or CDK6. Cyclin E is expressed later than the D-type cyclins in G1 phase and enters into a complex with CDK2. In contrast to the levels of cyclins, the expression of CDKs is not altered during the cell cycle. Cyclin-bound CDKs are phosphorylated on a single threonine residue by a CDK-activating kinase (CAK) and become catalytically active kinase complexes. Thus the expression of cyclins and the phosphorylation of cyclin-bound CDKs by CAK control the G1 cyclin-CDK kinase activity (Morgan,1995).

1.9.2 Negative regulation of G1 cyclin-CDK acivity

There is an additional mode of cell cycle regulation by CDK-inhibitory proteins (CKIs) which negatively regulate G1-S phase progression (figure 3). So far, seven CKIs have been found and can be divided into two major classes based on sequence similarity and mode of action. Members of INK4 family of inhibitors specifically bind to CDK4 and CDK6 and inhibit their association with cyclin D. There are four members of this family $p15^{Ink4B}$, $p16^{Ink4A}$, $p18^{Ink4C}$ and $p19^{Ink4D}$ (Sherr and Roberts, 1995). The Cip/Kip family of inhibitors comprises

three distinct gene products: $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. They bind to G1 cyclin-CDK complexes and inhibit their catalytic activity. These three proteins share an amino-terminal conserved region that is shown to be important for interaction with cyclin-CDK complexes (Lees, 1995; Massague´ and Polyak, 1995 and Sherr and Roberts, 1995).

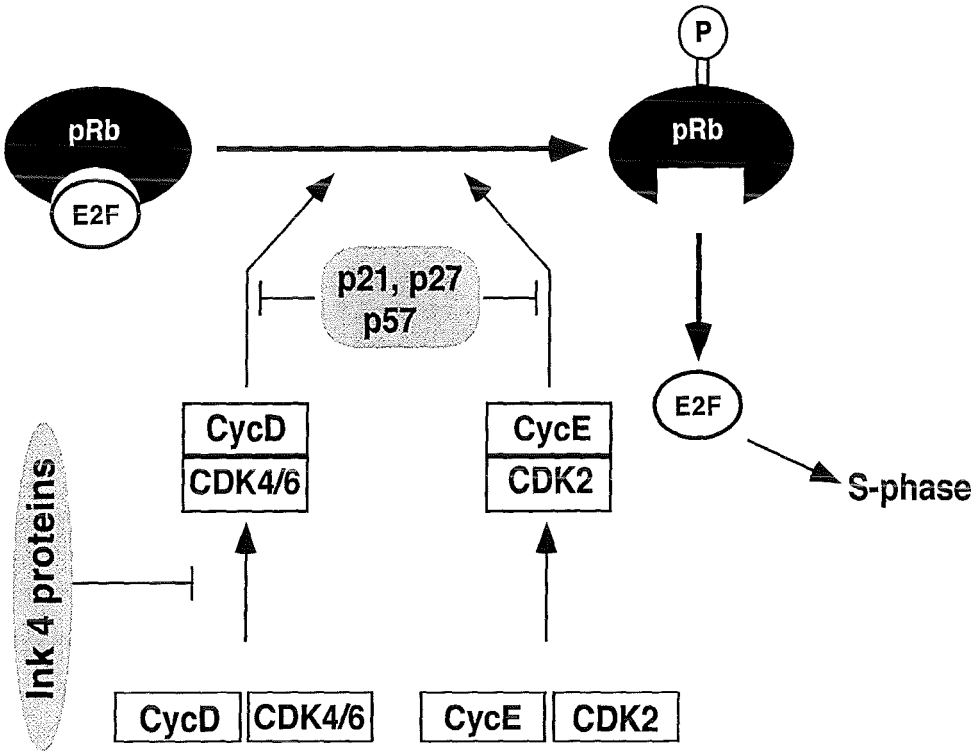

Figure 3. Regulation of G1 to S phase progression. The retinoblastoma tumor suppressor gene product (pRb) plays a key role in G1-S progression. In its hypophosphorylated state it binds to and negatively regulates transcription factors such as E2F, whose functions are important for G1- to S-phase progression. D-type cyclins (CycD) and cyclin E (CycE) acting together with cyclin-dependent kinases (CDKs), phosphorylate pRb during mid to late G1 phase. pRb releases E2F in response to phosphorylation. The liberated E2F enhances expression of genes required for S-phase entry. Cyclin-Cdk activity is negatively regulated by two families of CDK-inhibitory proteins (shaded). Ink4 type inhibitors bind to CDK4/6 and inhibit their association with cyclin D. The Cip/Kip family of inhibitors consisting of p21, p27 and p57 bind to cyclin-Cdk complexes and inhibit their catalytic activity (Sherr and Roberts, 1995).

The large number of inhibitors may reflect the requirement to respond to a variety of different signalling pathways. Transforming growth factor β (TGFβ)-induced cell cycle arrest for example is associated with increased levels of $p27^{Kip1}$ and $p15^{Ink4B}$ (Hannon and Beach, 1994; Polyak et al., 1994a and 1994b and Reynisdottir et al., 1995). $p27^{Kip1}$ is also identified as a key regulator of growth arrest in response to removal of growth factors (Coats et al., 1996). $p21^{Cip1}$ is a target of transcriptional activation by the p53 tumor suppressor gene product and is induced in response to DNA damage (Dulic et al., 1994 and El-Deiry et al., 1993). Expression of $p21^{Cip1}$ is also increased in senescing cells (Noda et al.,1994). The Cip/Kip proteins are also upregulated in many differentiating tissues suggesting that these proteins play a role in withdrawal from the proliferative cycle with the onset of differentiation (Sherr and Roberts, 1995).

Each CKI may play independent and non redundant roles in whole the organism as knock-out mice of each CKI revealed different phenotypes (Zavitz and Zipursky, 1997).

1.10 Aims

Dioxins are potent mammalian toxins affecting poliferation and differentiation of cells. Despite the knowledge that AhR mediates these effects, little is known about the signalling pathways which lead to dioxin toxicity. For studying dioxin toxicity, I used 5L rat hepatoma cells as a model system in which TCDD severely delays cell cycle progression through the G-1 phase in an AhR-dependent fashion. The aim of this work was to determine how AhR mediates TCDD-induced cell cycle delay. The following questions were addressed.

1. Is AhR dependently induced transcription of yet to be defined target genes required for TCDD induced cell cycle delay?

2. If AhR mediated transcription is required, what are target genes of AhR responsible for cell cycle effects and possibly the other toxic activities of dioxins?

   Two approaches were followed to find AhR regulated genes. One was the analysis of known cell cycle regulatory and, in particular, inhibitory proteins after TCDD treatment of 5L cells. The second approach was to find TCDD-induced genes by differential screening. Two of the identified genes were studied in detail for their role in TCDD toxicity.

3. Does the TCDD induced cell cycle delay follow the same AhR dependent signalling pathway as the known target gene in TCDD-induced metabolism of xenobiotica? In particular, the role of the AhR partner protein Arnt was tested.

2. Results

2.1 Functions of AhR which are required for cell cycle delay in 5L cells

One of the aims of the work presented here, was to determine whether AhR regulated gene transcription is required for TCDD-induced cell cycle delay. DNA binding and transactivation properties of AhR are required for transcriptional activation of genes (Hankinson, 1995). The BP8 cells are insensitive to TCDD due to the lack of AhR. Ectopic expression of AhR reconstitutes TCDD effects such as cell cycle delay in these cells (Weiß et al., 1996). The expression of various receptor mutants in BP8 cells allows to determine whether DNA binding and transactivation properties of AhR are required to reconstitute cell cycle delay.

2.1.1 Mutational analysis of AhR

Mutants of AhR which were defective in DNA binding or transactivation were created. This was to determine the functions of AhR required for reconstitution of cell cycle delay in the receptor deficient BP8 cells. A schematic outline of AhR and AhR mutants is given in figure 4. AhR R39A encodes an AhR with a single amino acid exchange at position 39 from arginine to alanine. This mutation was shown to be sufficient to abolish DNA binding to a XRE sequence both *in vivo* and *in vitro* (Dong et al., 1996). In GalAhR 83-805, the entire DNA binding domain of AhR is exchanged with that of the yeast Gal4 transcription factor and thus this mutant lacks the native AhR DNA binding activity but can activate gene expression from a Gal4-responsive promoter (Weiß et al., 1996). AhR 1-408 and AhR 1-605 encode receptors that partially (AhR 1-605) or completely lack the C-terminal transactivation domain.

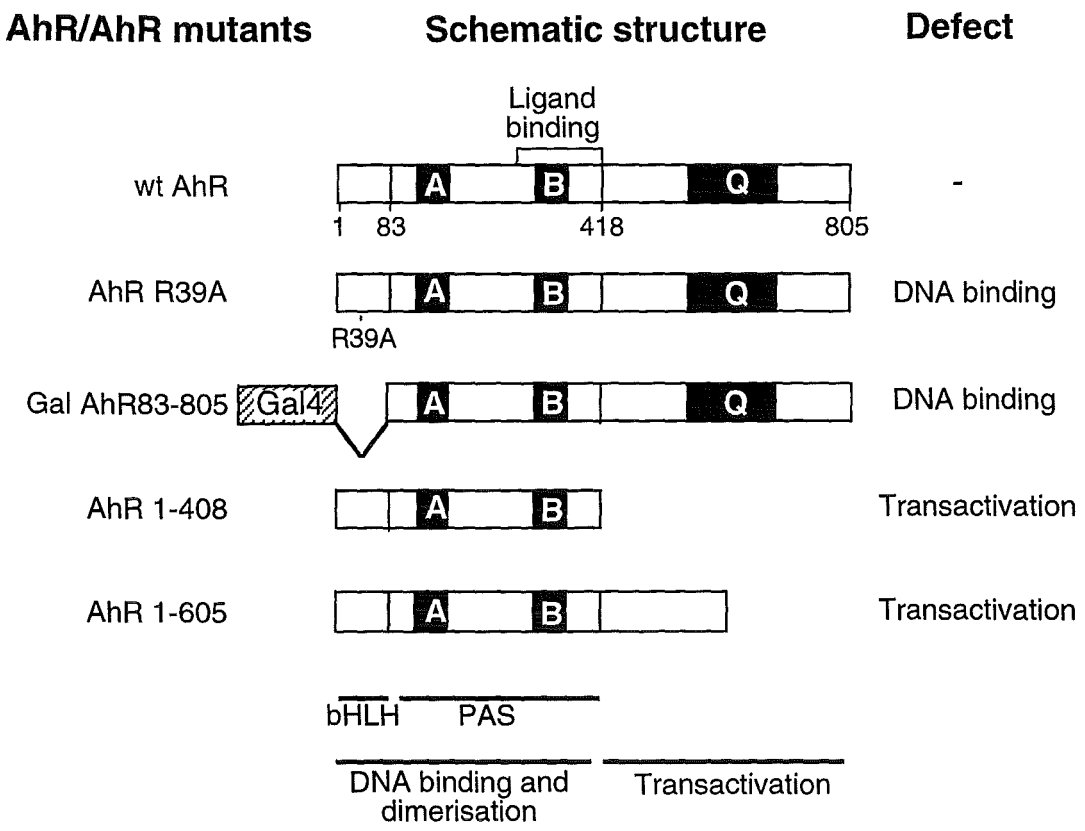

Figure 4. Schematic outline and expected defects of Ah receptor mutants used in this study in comparison to wild type Ah receptor (AhR). Numbers refer to the positions of amino acids. Marked areas identify the core bHLH-DNA-binding domain (1-83), the ligand binding domain and the conserved PAS-A (A) and PAS-B (B) motifs within the dimerization domain (83-418) and a glutamine-rich region (Q) within the transactivation domain (418-805).

DNA binding and transactivation domains of AhR are essential for transactivation of XRE regulated genes (Hankinson, 1995). Thus AhR mutants defective in DNA binding or transactivation should not transactivate a XRE-dependent reporter gene. To test whether the AhR mutants behave as expected, they were transiently cotransfected with a XRE-dependent luciferase reporter construct into the receptor deficient BP8 cells (figure 5). In BP8 cells the reporter gene was not inducible in the absence of AhR. Transfection of an expression vector for the full length receptor led to high basal luciferase activity without addition of TCDD and was further inducible two fold by TCDD treatment consistent with published data (Weiß et al., 1996). The DNA binding defective AhR mutant (AhR R39A) and the two C-terminal deletion constructs lacking the transactivation domain (AhR 1-408) or a part of it (AhR 1-605) did not activate the XRE-dependent reporter gene activity significantly.

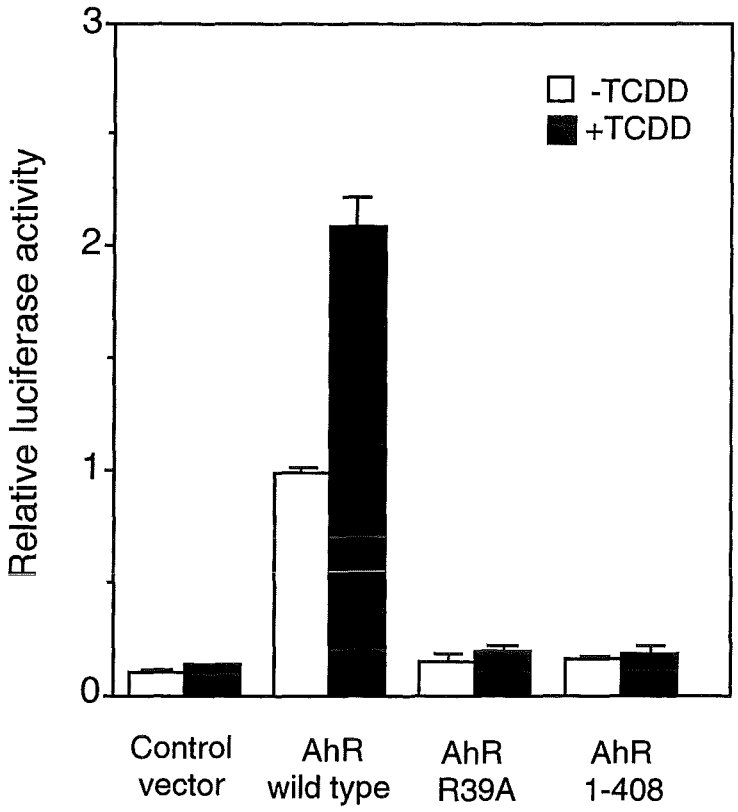

Figure 5. Transactivation of a XRE-dependent reporter gene by AhR or AhR mutants in AhR deficient BP8 cells. AhR deficient cells were transiently transfected by electroporation with 10 µg of the XRE-Luc reporter gene together with 2 µg of the expression vectors for AhR or AhR mutants or the empty expression vector as a control. Cells from the two electroporations transfected with the same DNA were pooled and then split equally onto two culture dishes. One was treated with 1 nM TCDD (filled bars) and the second with 0.1% DMSO (used as a solvent, open bars) immediately after transfection. Reporter gene activities were determined 24 h later. The reporter gene activity obtained with coexpression of the wild type AhR (solvent treated) was arbitrarily set to 1. Bars represent means +/- range from two independent experiments.

Functional expression of the AhR mutants was to be confirmed as they did not activate the XRE-dependent reporter activity. This was shown by transient cotransfection of various AhR mutants with the XRE-dependent reporter gene into 5L cells which contain endogenous AhR. If the two DNA-binding defective AhR mutants (AhR R39A and GalAhR 83-805) are functionally expressed, they should heterodimerize with the AhR's partner protein Arnt and by that sequester Arnt into complexes which can not bind to an XRE. This should limit the amounts of Arnt which are available to heterodimerize with endogenous AhR to form AhR-Arnt heterodimers binding to and activating the XRE-dependent reporter gene. If the two C-terminal deletion constructs lacking the complete transactivation domain (AhR 1-408) or a part of it (AhR 1-605) are functionally expressed, they

should heterodimerize with endogenous Arnt by forming mutant AhR-Arnt heterodimers with reduced transcriptional activity. These transcriptionally inactive heterodimers should compete with endogenous AhR-Arnt heterodimers for the XRE-binding sites on the reporter gene construct and as a result interfere with the expression of the reporter gene.

The results of such a cotransfection experiment are shown in figure 6. The XRE-dependent reporter gene was 15 fold inducible by TCDD in 5L cells without transfection of AhR mutants. Coexpression of various receptor mutants down regulated the reporter gene activity. This indicated that the AhR mutants could be functionally expressed.

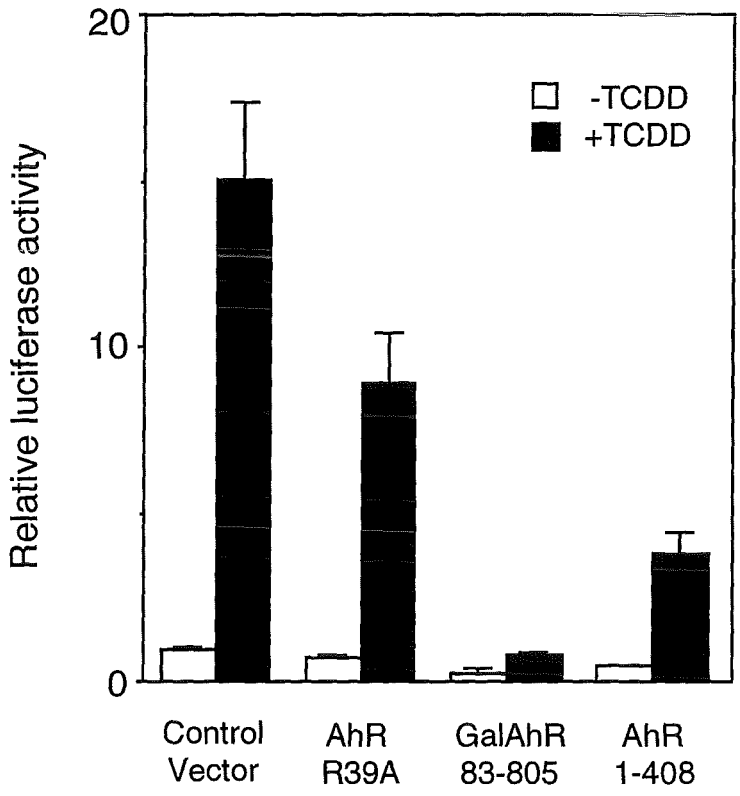

Figure 6. Expression of AhR mutants interferes with XRE-dependent reporter gene activity. 5L cells were transfected by electroporation with 6 µg of the XRE-Luc reporter construct and 24 µg of the expression vectors for AhR mutants or the empty expression vector as control. Cells from two electroporations transfected with the same DNA were pooled and then split equally onto two culture dishes. One dish was treated with 1 nM TCDD (filled bars) and the second with 0.1% DMSO (used as solvent, open bars) 12 h after transfection. Reporter gene activities were determined 40 h later. The reporter gene activity obtained with coexpression of the control vector (solvent treated) was arbitrarily set to 1. The bars represent means +/- range from 2 independent experiments.

2.1.2 Establishment of conditions in which transiently expressed mutants of AhR can be tested for their effect on cell proliferation

The mutants of AhR described in the previous paragraph were to be expressed in BP8 cells to determine whether DNA binding and transactivation properties of AhR are required to induce cell cycle delay. Attempts to generate stable transfectants using BP8 cells had failed before (Göttlicher - personal communication). Therefore a method was established to test the effect of AhR mutants on cell cycle by transient transfections. In transient transfections, only a minor population of cells efficiently takes up the transfected genes. Therefore the efficiently transfected cells had to be detected by some kind of 'flag' amongst the inefficiently transfected cells from the same culture dish. This would allow to analyse all cells simultaneously for their distribution over the cell cycle by flow cytometry and to use the 'flag' afterwards to gate the acquired data for efficiently transfected cells or inefficiently transfected cells respectively.

A method was developed employing coexpression of green fluorescent protein (GFP) (Chalfie., 1994 and Heim et al., 1995), which allowed identification of efficiently transfected cells by means of their green fluorescence in a flow cytometer (figure 7A).

To show that the system works, full length AhR was transiently coexpressed together with GFP in the AhR deficient BP8 cells. TCDD treatment reduced the proliferation of cells (represented by the decrease in the percentage of cells in the S and G2 phases of the cell cycle) in the efficiently transfected cells (green flourescing) expressing wild type AhR compared to the inefficiently transfected (non-green) cells of the same culture dish (figure 7, compare panels in row 2). When empty expression vector was transfected there was no effect of TCDD either in the efficiently transfected (green) compared to inefficiently transfected (non-green) population of cells (figure 7, compare panels in row 1 and the data for non transfected control vs. TCDD treated cells not shown). Thus the transient expression of full length receptor reconstitutes TCDD-induced cell cycle delay in BP8 cells.

2.1.3 Reconstitution of cell cycle delay by AhR mutants in the AhR deficient BP8 cells

The AhR mutants described in section 2.1.1 were tested for their ability to reconstitute cell cycle delay in the receptor deficient BP8 cells by the GFP cotransfection analysis. Neither the DNA binding defective (AhR R39A, GalAhR 83-805) nor the transactivation deficient AhR mutants (AhR 1-408, AhR 1-605) could reconstitute the cell cycle delay in BP8 cells.

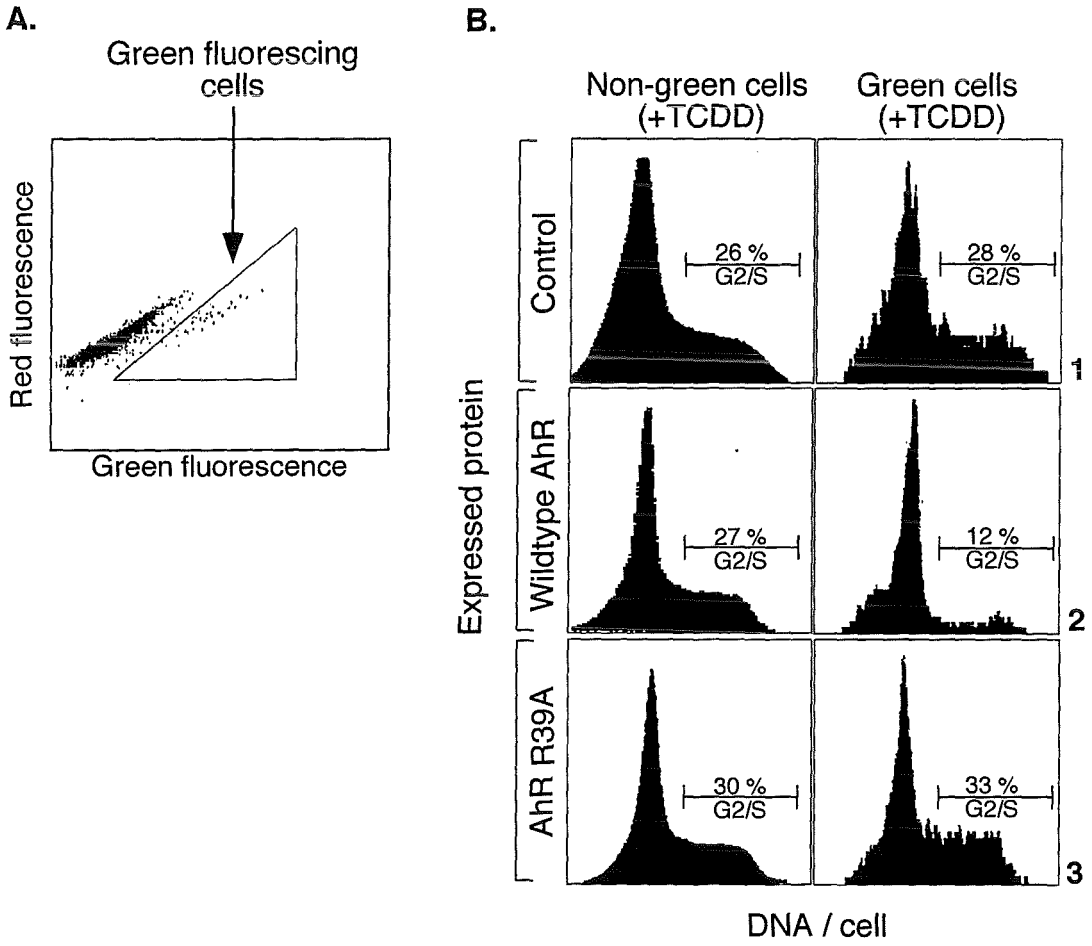

Figure 7. A DNA binding defective mutant Ah receptor (AhR R39A) does not reconstitute cell cycle delay in receptor deficient BP8 cells. BP8 cells were transiently cotransfected with 2.5 µg of expression vector for green fluorescent protein (GFP) and 24 µg of expression vectors for wild type AhR or mutant AhR (AhR R39A) or the empty expression vector as control. (**A**) The GFP-expressing sub population of transfected cells was identified by high green fluorescence as indicated in comparison to non-transfected cells by flow cytometry. (**B**) Cells were treated with 1 nM of TCDD, 24 h after transfection and harvested for flow cytometric analysis 24 h later. Cell cycle profiles were determined by H33258 staining and analysed individually for the green fluorescing sub populations and the non-green fluorescing sub populations. One representative out of 3 similar experiments is shown.

As an example, the cell cycle profiles obtained with the transient coexpression of the DNA binding defective AhR mutant (AhR R39A) and GFP are shown in figure 7. There was no effect of TCDD in the efficiently transfected (green) compared to inefficiently transfected (non-green) population of cells (figure 7 compare panels in row 3). The results obtained with other mutants are summarized in table I.

TABLE I

Reconstitution of cell cycle delay in the receptor deficient BP8 cells by expression of AhR or AhR mutants.

Name of the mutant	Defect	Cell cycle delay
Full length AhR (aa 1-805)	-	+
AhR R39A	DNA binding	-
GalAhR 83-805	Native DNA binding	-
AhR 1-408	Transactivation	-
AhR 1-605	Transactivation	-

The analysis of AhR mutants indicates that both DNA binding and transactivation domains of the AhR are essential for reconstitution of TCDD-induced cell cycle delay in BP8 cells. This suggested that transcriptional activation of genes by AhR is required for mediating cell cycle effects of TCDD, since it is unlikely that potential other modes of AhR action were as sensitive to all the mutations tested here.

There is further support for this hypothesis generated by Carsten Weiß (Personal communication). TCDD inhibits G1-phase progression prior to hyperphosphorylation of the retinoblastoma protein (Rb) in 5L cells synchronously released from a low serum-induced cell cycle arrest. Addition of TCDD 2 h prior to the expected hyperphosphorylation of Rb almost completely prevented Rb hyperphosphorylation. Treatment with the transcriptional inhibitor actinomycin D alone during these 2 h did not prevent Rb hyperphosphorylation. If TCDD was added together with actinomycin D, phosphorylated Rb accumulated as efficiently as in untreated cells. This indicated that the effects of dioxin on cell cycle require ongoing and presumably induced gene expression.

2.1.4 Dissociation of the XRE dependent induction of genes and cell cycle delay in 5L cells

Having established that the transcriptional activation of genes by AhR is required for mediating cell cycle effects of dioxins, I now asked whether activation of XRE-regulated genes could be essential for these effects.

For this purpose I compared different ligands or doses of TCDD for their ability to induced XRE-regulated transcription and cell cycle delay.

TCDD induced *CYP450 1A1*, a classical XRE-regulated gene, and delayed cell cycle progression at 1 nM concentration (figure 8A, compare lanes 1 and 4). However, TCDD at lower concentrations (1 and 5 pM) induced *CYP450 1A1* but did not have any effect on the cell cycle progression (figure 8A, compare lanes 1, 2 and 3). Thus induction of *CYP450 1A1* and cell cycle delay mediated by AhR follow different dose response relationships.

Among AhR binding dioxins there is a good correlation between the affinity of a given ligand, and its potency to induce transcription of the *CYP450 1A1* gene in vivo (Berghard et al.,1992). However, there are few compounds distinct from dioxins which bind to the receptor but are poor activators, so-called partial agonist/antagonists (Wilhelmsson et al., 1994). One of these compounds, α-naphthoflavone (ANF), was tested for its ability to induce XRE-regulated transcription and cell cycle delay (figure 8B). ANF induced the cell cycle delay in 5L cells without increasing *CYP450 1A1* or *GST-Ya* transcription after 24-96 hours of treatment (figure 8B, compare lanes 1 to 5). The effect of ANF is AhR dependent, as it did not have any effect on the proliferation of receptor deficient BP8 cells (data not shown). Thus the induction of *CYP450 1A1* or *GST-Ya* representing XRE regulated genes in two promoter contexts was not essential for cell cycle delay. These data also suggest that the induction of XRE-dependent genes and the cell cycle effects of TCDD may involve qualitatively distinct modes of AhR action. The AhR activates transcription of XRE-regulated genes as a heterodimeric complex with Arnt. Thus it is possible that the AhR-Arnt complex which binds to the classical XRE may not be essential for TCDD-induced cell cycle delay.

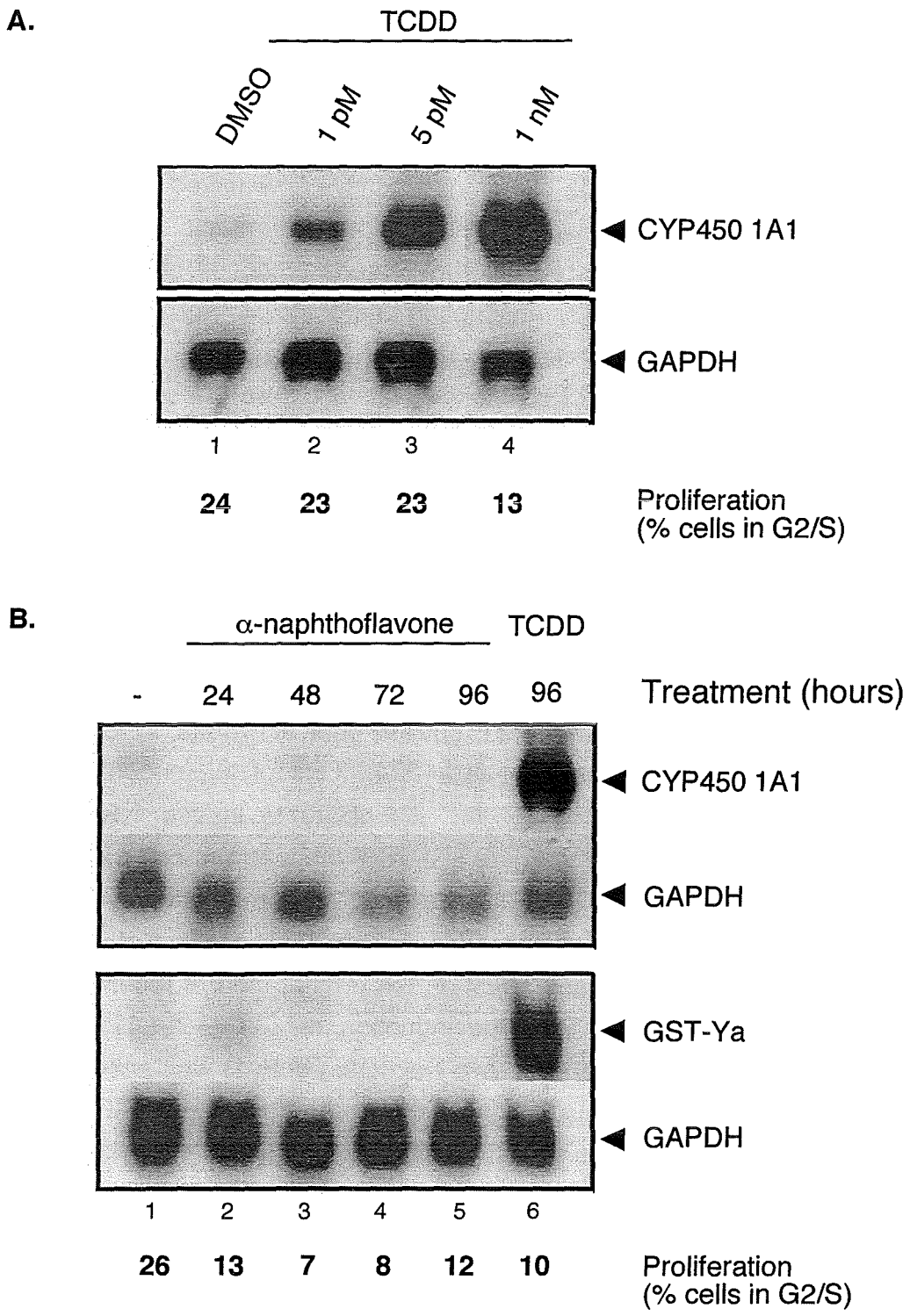

Figure 8. Dissociation of AhR-mediated cell cycle delay and induction of XRE-regulated genes in 5L cells. 5L cells were treated with (**A**) various concentrations of TCDD for 24 h or (**B**) with 20 μM α-naphthoflavone or 1 nM TCDD for the indicated time interval. Cell proliferation (% of cells in G2/S phase) was estimated from the cell cycle profiles obtained with H33258 staining of cells by flow cytometry. Total RNA was prepared from parallel culture dishes and induction of (**A**) CYP450 1A1 or (**B**) CYP450 1A1 and GST-Ya RNAs was tested by Northern blot analysis using 10 μg total RNA. Equal loading of RNA was confirmed by rehybridisation of the blots with a GAPDH probe.

2.1.5 Arnt but not mutant Arnt antagonises TCDD-induced cell cycle delay in 5L cells

To determine whether Arnt is required for the cell cycle delay mediated by AhR, mutant Arnt was used which is defective in DNA binding. When over-expressed, this mutant is expected to heterodimerize with endogenous AhR and to sequester AhR into non-DNA binding inactive heterodimers with the mutant Arnt. If activation of XRE regulated genes were required for TCDD-induced cell cycle delay, overexpression of the DNA binding defective mutant Arnt (ArntΔbHLH) should interfere with the cell cycle delay. Similarly, overexpression of Arnt is expected to synergise with the TCDD-induced cell cycle delay if the cell cycle delay depends on upregulation of genes by the AhR-Arnt heterodimer.

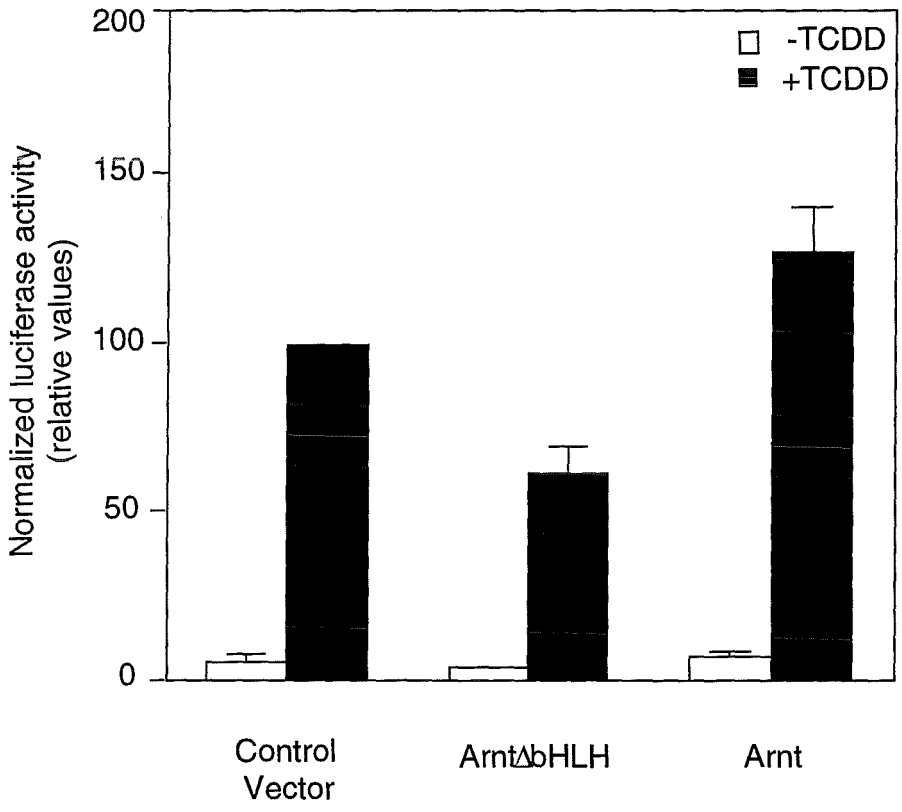

Figure 9. Effect of Arnt and mutant Arnt overexpression on XRE-dependent reporter gene activity. 5L cells were transfected with 6 µg of the XRE-Luc reporter gene and 10 µg of expression vectors for Arnt or mutant Arnt or the empty expression vector as control. Cells from two electroporations transfected with the same DNA were pooled and then split equally onto two culture dishes. One dish was treated with 1 nM TCDD (filled bars) and the second with 0.1% DMSO (used as solvent, open bars) 12 h after transfection. Reporter gene activities were determined 40 h later and values were normalised for the activity of a 2 µg of a cotransfected expression vector for Renilla luciferase. The bars represent means +/- S.D. from 3 independent experiments.

The behaviour of Arnt and the DNA binding defective mutant Arnt (ArntΔbHLH) was first tested in a reporter gene assay by transient transfection. Arnt and ArntΔbHLH were cotransfected with a XRE-dependent luciferase reporter gene construct into 5L cells which contain endogenous AhR and Arnt. The coexpression of Arnt had a positive effect probably because endogenous levels are limiting and ArntΔbHLH had the expected dominant negative effect on the reporter gene activity (figure 9).

Next, full length Arnt or DNA binding defective mutant Arnt (ArntΔbHLH) were transiently coexpressed with GFP in 5L cells to determine whether Arnt was required for the TCDD-induced cell cycle delay. Cell cycle analysis of the efficiently transfected (green) population of cells by flow cytometry revealed that expression of the full length Arnt prevented TCDD-induced cell cycle delay (figure 10, compare 5 and 6), whereas the ArntΔbHLH had no effect (figure 10 compare (8 and 9). These effects of Arnt and mutant Arnt are the opposite to what would be expected from a phenomenon which depends on upregulation of genes by the AhR-Arnt heterodimer. Arnt would have synergised with the AhR and the DNA binding defective Arnt mutant (ArntΔbHLH) should have acted dominant negatively. These data suggest that the AhR/Arnt heterodimer is not required for TCDD-induced cell cycle delay. Possibly AhR acts without a partner protein or together with partner protein(s) distinct from Arnt.

In summary, the most likely interpretation of the experiments in this section is that transcriptional activation of genes is required for TCDD-induced cell cycle delay. AhR mediated cell cycle delay seems, however, not to depend on gene activation by AhR/Arnt heterodimer. AhR regulated genes which are likely to act on cell cycle were not known. Hence most of the remaining work was dedicated to finding such AhR regulated genes for cell cycle control.

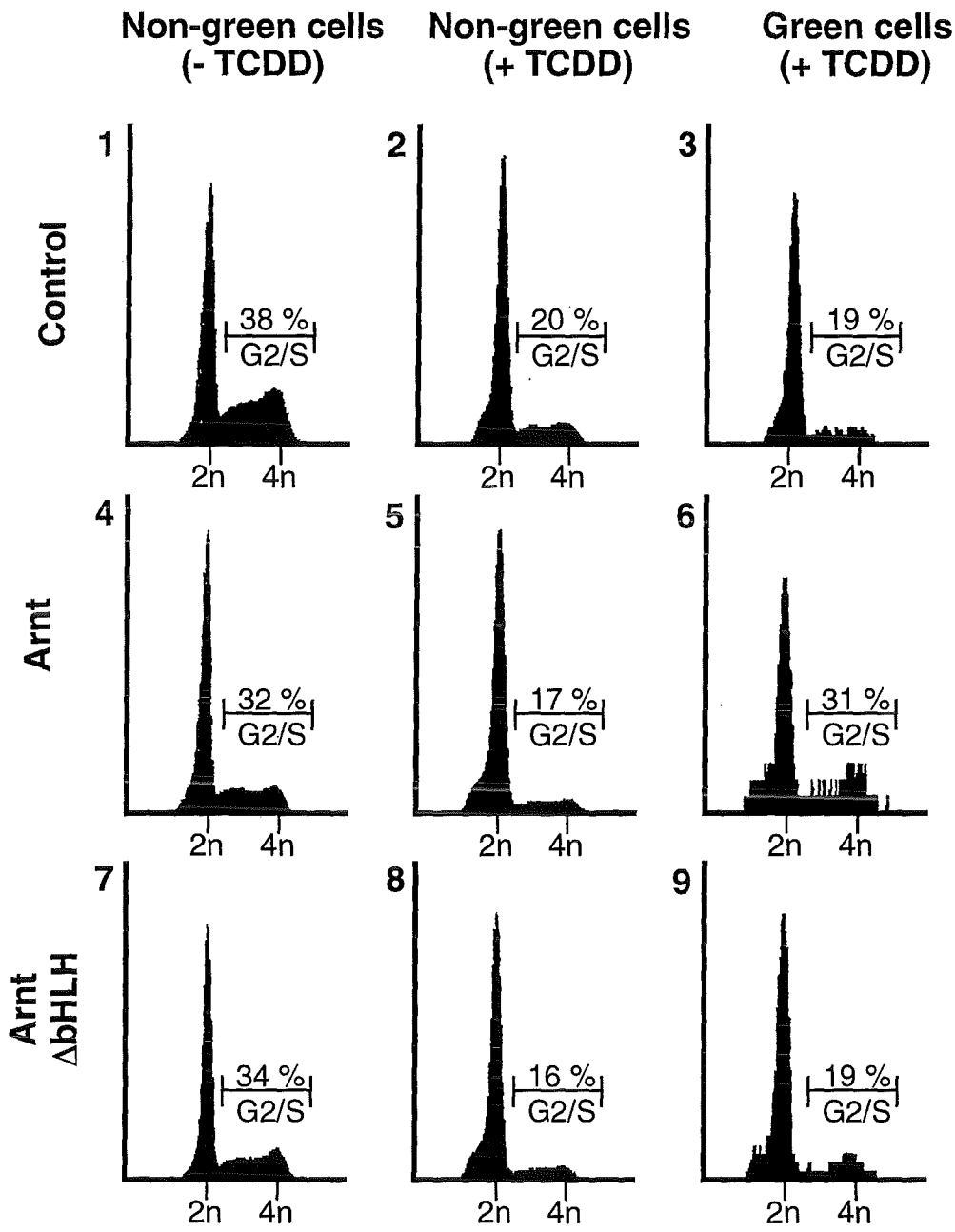

Figure 10. Arnt but not DNA-binding deficient Arnt antagonises TCDD-induced cell cycle delay in 5L cells. 5L cells were transiently cotransfected with 2.5 µg of expression vector for green fluorescent protein (GFP) and 10 µg of expression vectors for wild type Arnt or DNA binding deficient Arnt (Arnt ΔbHLH) or the empty expression vector as control. Cells were treated with 1 nM TCDD or 0.1% DMSO solvent 12 h after transfection and harvested for flow cytometric analysis 40 h later. The GFP-expressing subpopulation of transfected cells was identified by high green fluorescence in comparison to non-transfected cells. Cell cycle profiles were determined by H33258 staining and analysed individually for the transfected green fluorescing subpopulations (green) and the non-transfected (non-green) subpopulations.

2.2 Search for AhR regulated genes

Two approaches were followed simultaneously to find AhR regulated genes involved in TCDD-induced cell cycle delay. One of the approaches was to analyse the known inhibitor proteins regulating cell cycle progression. The second approach was to systematically search for AhR target genes by subtractive hybridization.

2.2.1 Cell cycle inhibitory proteins

Cell cycle progression is regulated through the co-ordinated expression, and degradation of cyclins and posttranslational modification of their catalytic partners, cyclin-dependent kinases (CDKs). Cyclins, expressed at particular stages of the cell cycle, associate with specific CDKs to form active kinase complexes which phosphorylate multiple cellular proteins like Rb and promote cell cycle progression. Cyclin-CDK complexes are negatively regulated by a family of CDK-inhibitory proteins (CKIs) which bind to CDKs or cyclin-CDK complexes and inhibit their catalytic activity, ultimately impeding cell cycle progression (Sherr and Roberts, 1995).

2.2.1.1 TCDD induces the $p27^{Kip1}$ cell cycle inhibitor

5L cells are arrested in G1 phase of the cell cycle upon TCDD treatment. The various cyclin/CDK inhibitors which could lead to a G1 arrest, were analysed in the laboratory and $p27^{Kip1}$ levels were found to be elevated after 4, 8 or 24 hours of TCDD treatment (figure 11A). The increase in protein levels coincided with the increase in Kip1 mRNA after 4 or 24 hours of TCDD treatment (figure 11B).

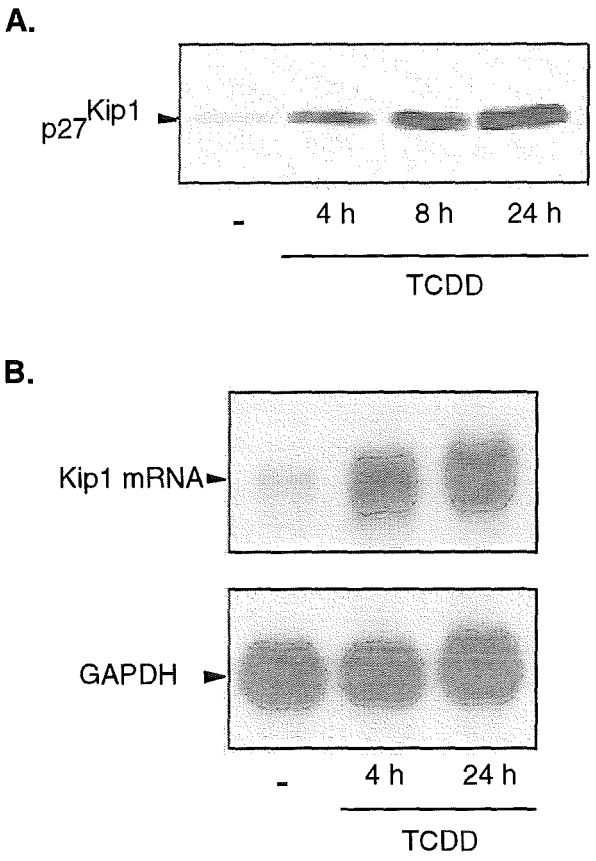

Figure 11. TCDD induces the $p27^{Kip1}$ cell cycle inhibitor. 5L cells were non-treated or treated with 1 nM TCDD for the indicated time interval. $p27^{Kip1}$ (**A**) protein levels were determined by Western blot analysis of whole cell extracts. Comparable loading of lanes was confirmed by Coomassie staining of a part of the gel. (**B**) mRNA levels were determined by Northern blot analysis using 10 µg of total RNA. Equal loading of RNA was confirmed by rehybridisation of the blot with a GAPDH probe.

To determine whether the increase in Kip1 mRNA levels was due to an increase in stabilization of mRNA by TCDD, the decay of Kip1 mRNA was followed in solvent or TCDD treated cells after disruption of transcription by the transcriptional inhibitor actinomycin D (figure 12). The half life of Kip1 mRNA in solvent or TCDD treated cells was found by quantitative evaluation to be about 100 and 110 minutes respectively (figure 12B). Thus stabilization of mRNA is not the cause for the increased levels of Kip1 steady state mRNA levels in TCDD-treated 5L cells.

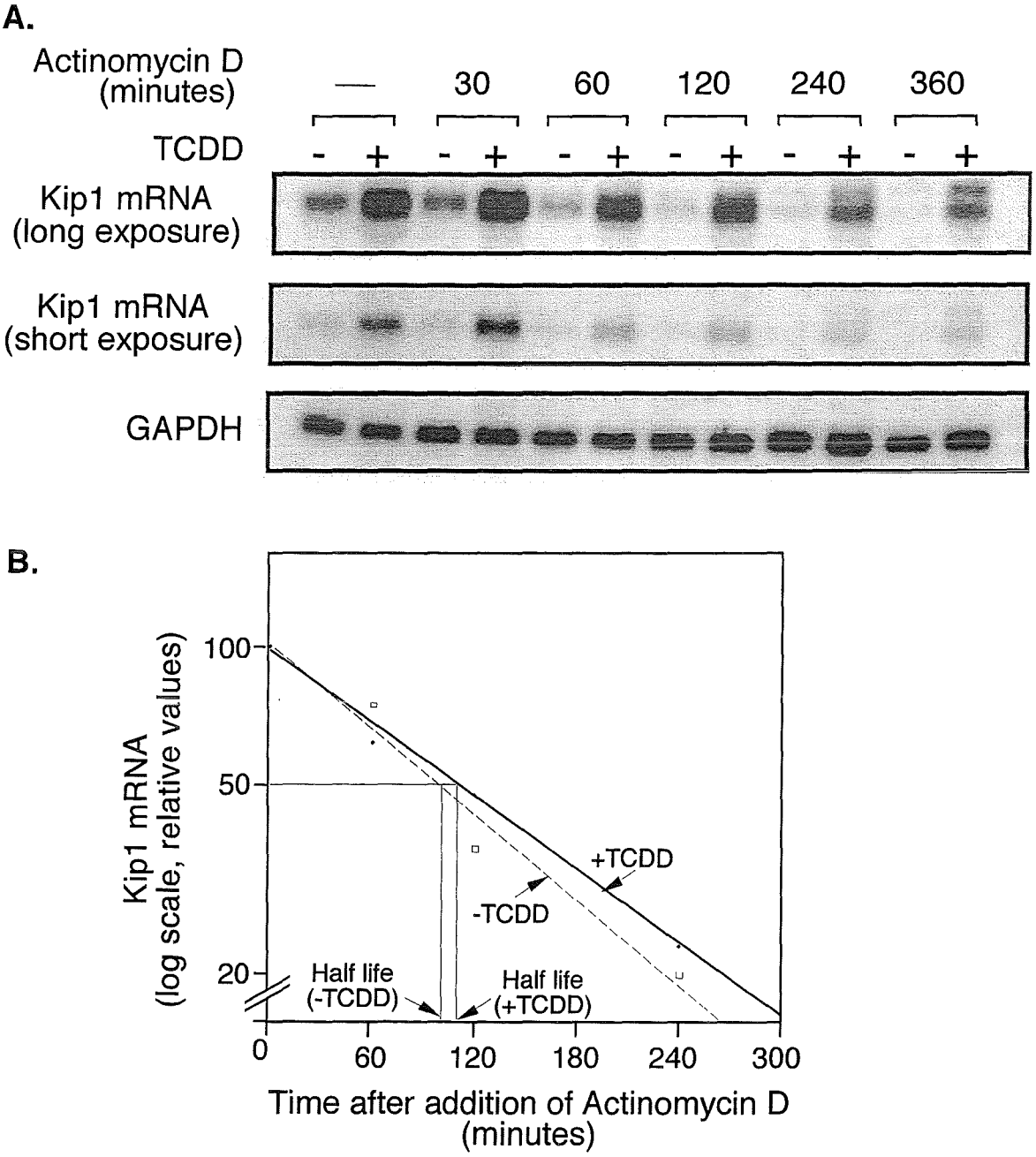

Figure 12. Kip1 mRNA half life in control and TCDD-treated 5L cells.
(**A**) Decay of Kip1 mRNA after inhibition of transcription by actinomycin D. 5L cells were treated with 1 nM TCDD or 0.1% DMSO solvent control. 4 h Later cells were additionally treated with actinomycin D (5 µg/ml) for the indicated time interval. Kip1 mRNA levels were determined by Northern blot analysis using 10 µg of total RNA. The blot was stripped and rehybridised with a GAPDH probe to correct for variations in RNA loading. (**B**) Graphic representation and quantitative evaluation of the data shown in (A) normalised to GAPDH mRNA amounts. The normalised Kip1 mRNA levels in the control/TCDD-treated cells without actinomycin D treatment were arbitrarily set to 100. The lines in figure represent the decay of Kip1 mRNA in the solvent (dashed line) and TCDD-treated (solid lines) cells. The thin lines indicate determination of the half life times. The half lives of Kip1 mRNA in the control and TCDD-treated 5L cells are estimated to be 100 and 110 minutes.

2.2.1.2 Kip1 mRNA induction by TCDD does not require new protein synthesis

It was determined whether Kip1 mRNA is induced by TCDD directly or as the consequence of induction of additional intermediary proteins. TCDD-induced Kip1 mRNA levels were analysed after inhibition of protein synthesis by cycloheximide pre-treatment (figure 13). Cycloheximide pre-treatment increased the level of Kip1 mRNA expression (compare lanes 1 and 3) for unknown reason. However, TCDD still induced the Kip1 mRNA on top of the increased levels obtained with cycloheximide treatment (compare lanes 3 and 4). Thus Kip1 mRNA induction by TCDD is direct and does not require expression of intermediary proteins. Thereby Kip1 is identified as a novel target gene of AhR.

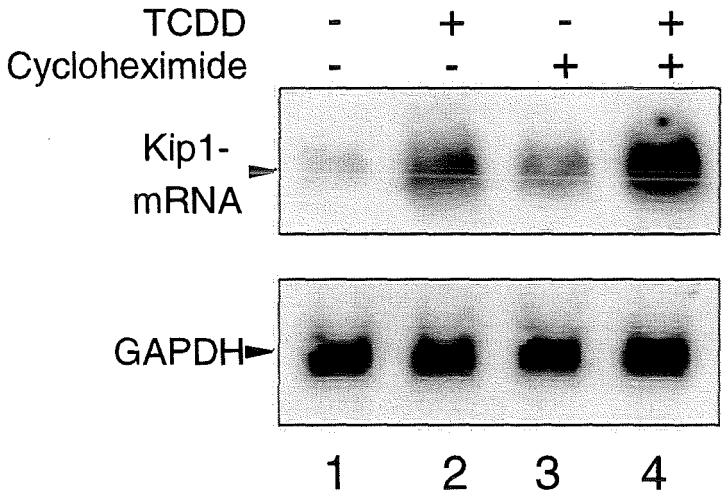

Figure 13. Kip1 mRNA induction by TCDD does not require new protein synthesis. Induction of Kip1 mRNA was tested after 4 h of exposure to 1 nM TCDD or 0.1% DMSO solvent with or without 30 min of pre-treatment with the translational inhibitor cycloheximide (50 µg/ml) by Northern blot analysis using 10 µg of total RNA. Equal loading of RNA was confirmed by rehybridisation of the blot with a GAPDH probe.

2.2.1.3 Comparison of dose dependent induction of CYP450 1A1 and Kip1 mRNAs by TCDD

To test whether the increase in Kip1 mRNA levels follow the increase in mRNA levels of classical XRE-regulated genes like *CYP450 1A1*, the dose response curves of Kip1 and *CYP450 1A1* mRNA induction were compared (figure 14).

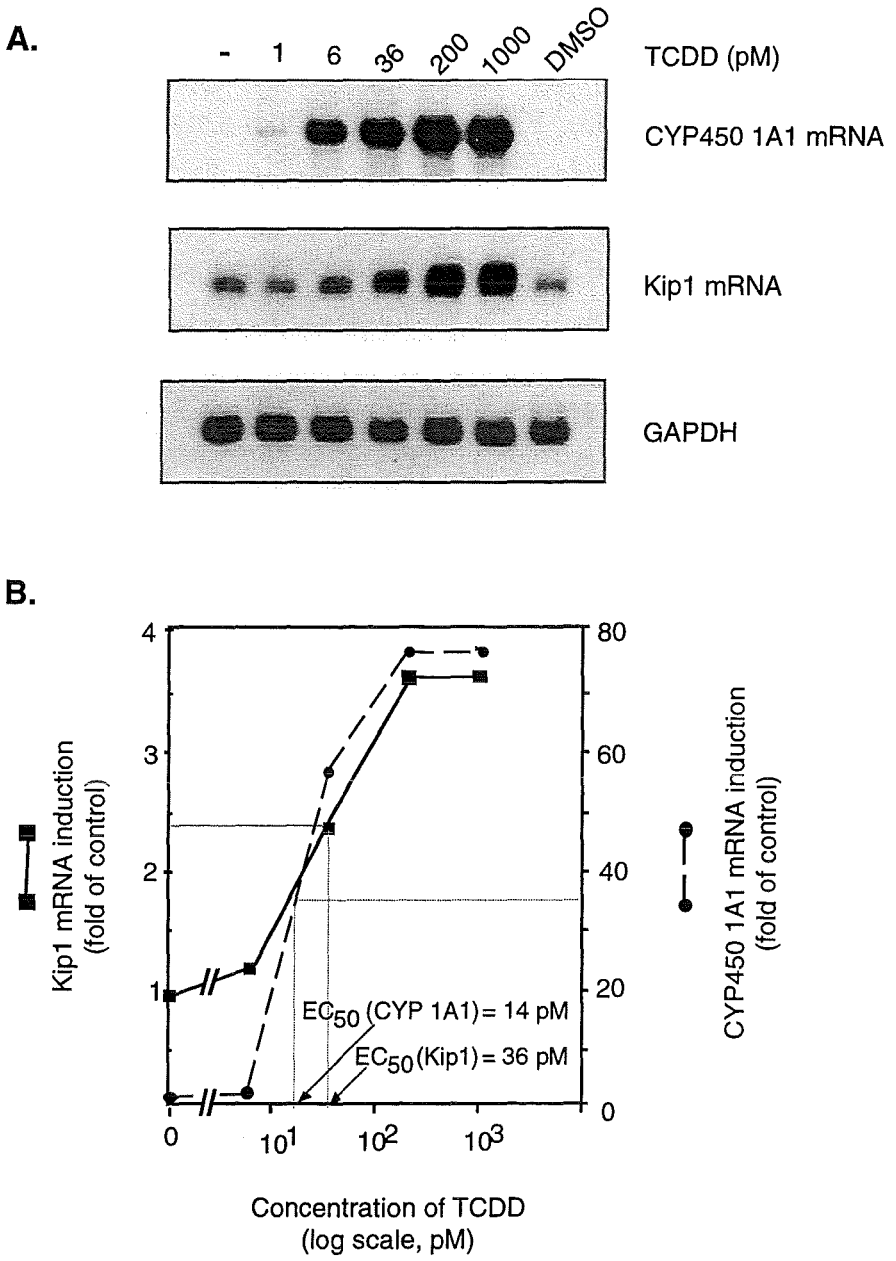

Figure 14. Dose dependent induction of CYP450 1A1 and Kip1 mRNAs by TCDD. **(A)** Northern blot analysis was done using 2 µg poly(A)+ RNA for CYP450 1A1 and Kip1 mRNAs after 24 h treatment with different doses of TCDD or 0.1% DMSO solvent control. Loading of RNA was controlled by rehybridisation of the blot with a GAPDH probe. Dose response curves for Kip1 and CYP450 1A1 mRNA induction are normalised to GAPDH shown in **(B)**. The TCDD doses for half-maximal induction (EC_{50}) of Kip1 and CYP450 1A1 mRNA are estimated to be 36 and 14 pM respectively.

The halfmaximal TCDD doses for Kip1 and CYP450 1A1 mRNA induction were calculated and found to be 36 and 14 pm respectively. Interestingly the halfmaximal response dose for TCDD-induced cell cycle delay is reported to be

30-100 pM (Göttlicher et al., 1990). Thus there is a correlation of the halfmaximal dose for $p27^{Kip1}$ mRNA induction and the cell cycle delay. This suggested that $p27^{Kip1}$ may be a relevant target gene for mediating cell cycle effects of TCDD in 5L cells.

2.2.1.4 Increased $p27^{Kip1}$ levels - Cause or consequence of TCDD-induced cell cycle delay?

Having shown that TCDD directly induces Kip1 without the need for induction of additional intermediary proteins still does not prove that Kip1 indeed is the cause of the cell cycle arrest. It also appears possible that TCDD arrests 5L cells in the G1-phase of the cell cycle and this particular segment of the G1 phase could per se be associated with high expression of Kip1. To rule out this possibility, 5L cells were arrested in the G1-phase of the cell cycle by serum starvation for 24 hours. These cells were treated for an additional 4, 8 and 24 hours in serum free medium with TCDD. Extracts from serum starved cells revealed that Kip1 protein and mRNA levels remained at low levels, even though cell cycle progression was delayed by serum starvation (figure 15, compare lanes 1 and 3). Thus, an arrest in G1-phase of the cell cycle did not necessarily lead to increased levels of Kip1 protein or mRNA. However, when the serum starved cells were treated with TCDD, both Kip1 mRNA and protein levels were increased (figure 15, compare lanes 4 to 5, 6 to 7 and 8 to 9). This indicated that increased $p27^{Kip1}$ levels upon TCDD treatment were not a consequence of a delay in cell cycle.

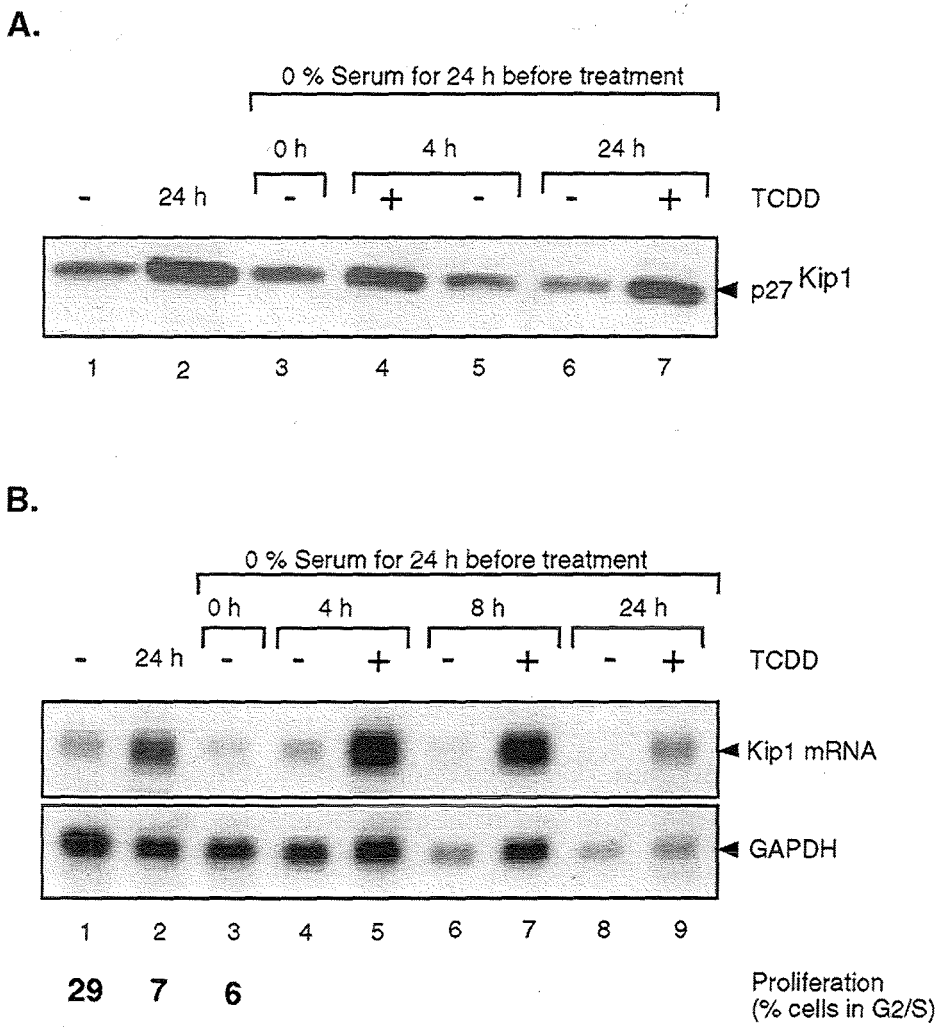

Figure 15. Induction of $p27^{Kip1}$ by TCDD in G1-arrested 5L cells. 5L Cells were arrested in G1 phase of the cell cycle by serum starvation for 24 h. Cells were then treated with 1 nM TCDD or 0.1% DMSO solvent control for the time intervals indicated. Non-serum starved cells were treated for 24 h with 1 nM TCDD or 0.1% DMSO. (**A**) $p27^{Kip1}$ protein levels were determined by Western blot analysis of whole cell extracts. Comparable loading of lanes was confirmed by Coomassie staining of a part of the gel. (**B**) mRNA levels were determined by Northern blot analysis using 10 µg of total RNA. Loading of RNA was controlled by rehybridisation of the blot with a GAPDH probe. Cell proliferation (% of cells in G2/S phase) was estimated from the cell cycle profiles obtained with H33258 staining of cells from parallel culture dishes treated with 1 nM TCDD or 0.1% DMSO for 24 h or 24 h serum starved cells.

2.2.1.5 Expression of antisense Kip1 RNA impairs TCDD-induced cell cycle delay

To now prove directly that $p27^{Kip1}$ is required for TCDD-induced delay of cell cycle progression, antisense Kip1 RNA was transiently expressed to inhibit the accumulation of Kip1 protein. Coexpression of GFP allowed identification of

the efficiently transfected cells by flow cytometry as described in section 2.1.2. Cell cycle profiles were determined by H33258 staining and analysed individually for the efficiently transfected green fluorescing subpopulations and the inefficiently transfected (non-green) subpopulations. If GFP was expressed together with an empty expression vector, TCDD treatment reduced the relative number of cells in S/G2 phase in both the efficiently transfected and the majority of the inefficiently transfected cells of the same culture dish (figure 16, compare panels in rows 1 and 2). If Kip1 antisense RNA was expressed together with GFP, the TCDD-effect on cell cycle distribution was disrupted in the efficiently transfected subpopulation of cells (figure 16, compare panels in row 3). In the inefficiently transfected subpopulation of the same cell culture dish, TCDD delayed cell cycle progression as expected (figure 16, compare panels in row 4).

In a second approach, bromodeoxyuridine (BrdU) incorporation was monitored after the cells were transfected with antisense Kip1 cDNA or empty expression vector. BrdU is an analogue of thymidine which is incorporated into actively proliferating cells. The efficiently transfected cells were isolated by fluorescence activated cell sorting (FACS) for the analysis of Kip1 protein expression and incorporation of BrdU in to S-Phase cells (figure 17).

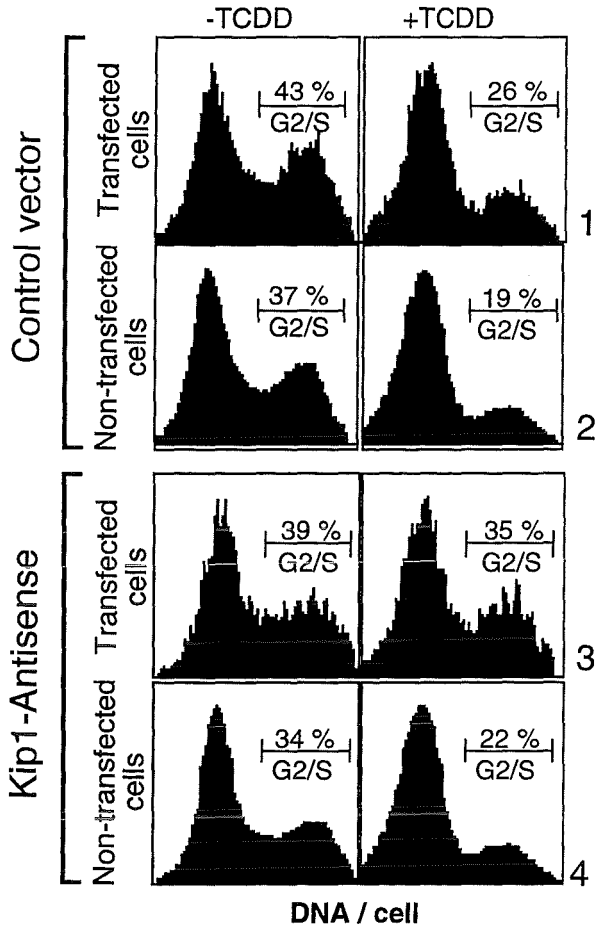

Figure 16. Expression of antisense Kip1 RNA impairs TCDD-induced cell cycle delay in 5L cells. 5L cells were transiently cotransfected with expression vectors for a Kip1 antisense RNA and the green fluorescent protein (GFP) or the empty expression vector and GFP. Cells were treated with 1 nM of TCDD or 0.1% of the DMSO solvent control 32 hours after transfection and harvested for flow cytometric analysis 18 hours later. The GFP-expressing subpopulation of transfected cells was identified by high green fluorescence in comparison to non transfected cells (please refer also to figure 4). Cell cycle profiles were determined by H33258 staining and analysed individually for the efficiently transfected green fluorescing subpopulations and the non-transfected (non green) subpopulations. One representative out of 4 similar experiments is shown.

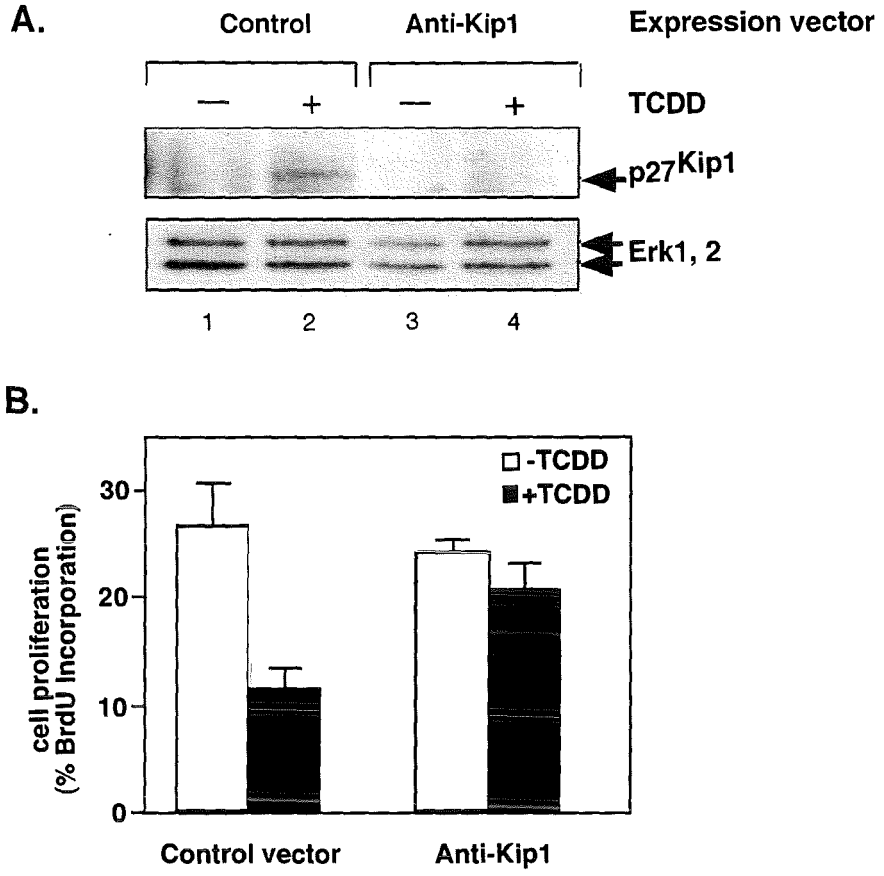

Figure 17. Expression of Kip1 antisense RNA inhibits the accumulation of TCDD-induced Kip1 protein and reverses the effects of TCDD on BrdU incorporation. Proliferation rates were determined by the incorporation of BrdU in 5L cells which had been transiently cotransfected with expression vectors for a Kip1 antisense RNA (Anti-Kip1) and the green fluorescent protein (GFP) or the empty expression vector (control) and GFP. Green fluorescing cells were collected by FACS sorting and were either (**A**) analysed by Western blotting for the expression of $p27^{Kip1}$ protein, or (**B**) spun onto glass slides for immunocytochemical detection (Kit by Boehringer, Mannheim) of the percentage of cells having incorporated BrdU. 32 h after transfection cells were treated with 1 nM of TCDD or 0.1% of the DMSO solvent control for additional 18 h of which during the last 5 h BrdU was present in the culture medium. Cells were harvested using trypsin and green fluorescing cells were sorted to a purity greater than 80%. For Western blot analysis $2x10^4$ cells were lysed in sample buffer. Comparable loading of lanes within a factor of 2 was confirmed by reprobing the blot against ERK1 and 2 and Coomassie staining of a part of the gel. Bars in part B represent means +/- S.D. from 3 independent experiments.

In cells transfected with GFP and the empty expression vector, TCDD induced Kip1 in the efficiently transfected (green) cells (figure 17A, compare lanes 1 and 2). TCDD also inhibited proliferation as indicated by the reduction of BrdU incorporation (figure 15B, compare the left pair of bars). After expression of Kip1 antisense RNA, no TCDD-induced Kip1 could be detected (figure 17A, compare lanes 3 and 4) and TCDD effects on Brdu incorporation were only marginal

(figure 17B, compare the right pair of bars). The minor inhibition of proliferation could be explained by 10% of non-transfected cells sticking to and copurifying with the sorted fluorescing cells as revealed by microscopic examination. These data strongly indicate that Kip1 is required for AhR-dependent delay of 5L cell proliferation.

In summary, TCDD induces Kip1 by inducing its mRNA. Kip1 mRNA induction is direct as the translational inhibitor cycloheximide had no effect. Kip1 plays a causal role in TCDD-induced cell cycle delay as inhibition of its accumulation reversed the effects of TCDD on the cell cycle.

2.2.2 Suppression subtractive hybridization to find target genes of AhR

In addition to the analysis of cell cycle regulatory proteins a systematic search for AhR induced genes in 5L cells was initiated for two reasons. (i) initially it was not clear which approach could be successful. (ii) not all activities of dioxins/AhR may follow the same pathway and need the same initially AhR regulated genes.

Subtractive hybridization is a powerful technique that allows comparison of two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. A new PCR-based cDNA subtraction method, termed suppression subtractive hybridization (SSH) has been described recently to obtain differentially expressed genes (Diatchenko et al., 1996; Gurskaya et al., 1996). SSH was used to search for AhR target genes in 5L cells.

2.2.2.1 The principle of suppression subtractive hybridization (SSH)

A schematic representation of the SSH method is shown in figure 18. Both the mRNA populations to be compared are first converted into cDNA. The cDNA that contains specific differentially expressed transcripts is referred as 'tester' and the reference cDNA as 'driver'. The driver and tester ds cDNAs are first digested with Rsa I, a four-base cutting restriction enzyme that yields blunt ends. After digestion cDNAs have an average length of approximately 600 bp, which aids hybridization of complementary strands. The tester cDNA fragments are then divided into two fractions and each is ligated with a different cDNA adaptor. The ends of the adaptor do not have a phosphate group, so only one strand of each

adaptor attaches to the 5' ends of cDNA and the adaptors cannot be ligated into dimers.

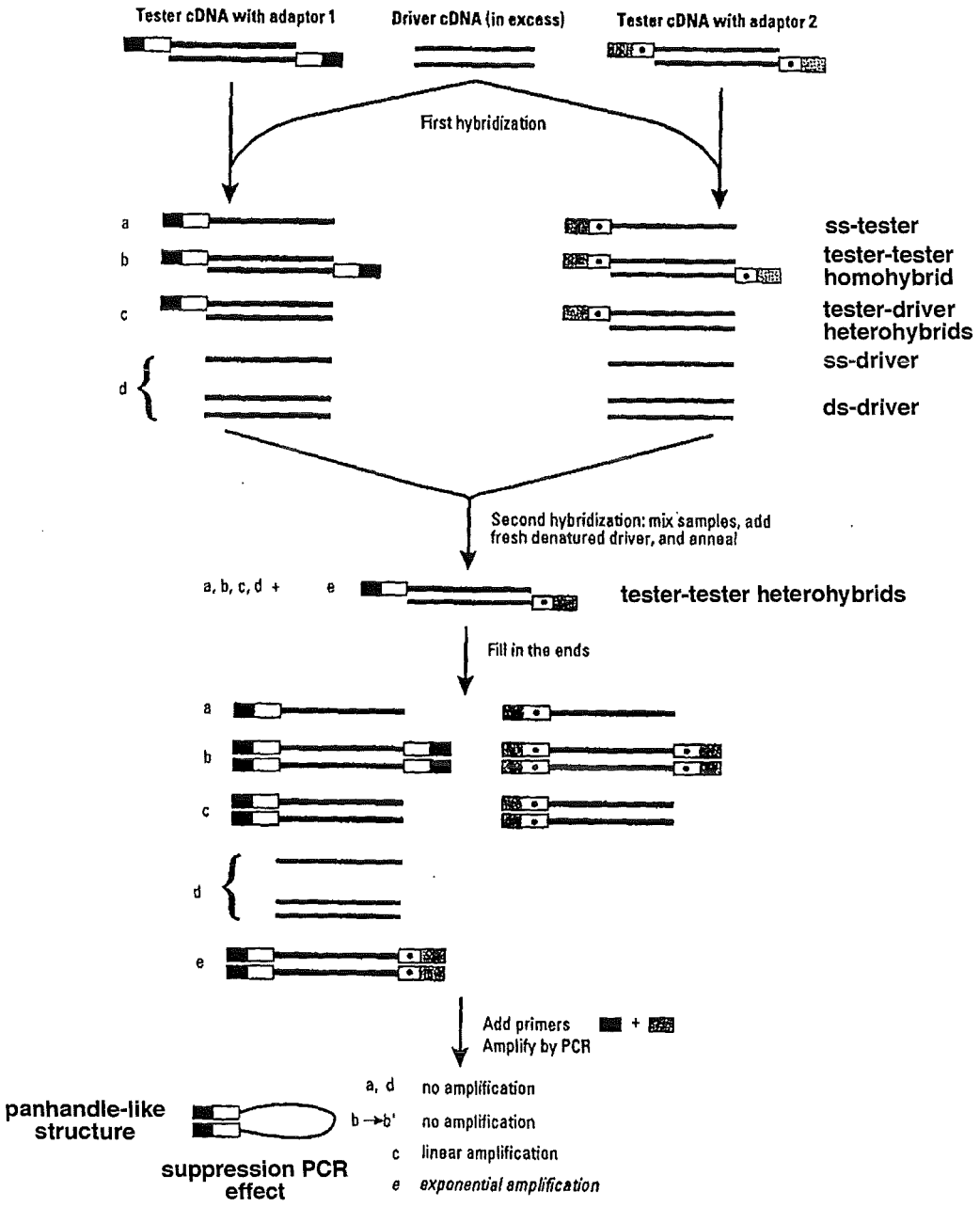

Figure 18. Scheme of Suppression Subtractive Hybridisation (SSH). Solid lines represent the RsaI digested tester or driver cDNA. Solid boxes represent the outer part of the adaptor 1 corresponding to PCR primer 1. Shaded boxes represent the outer part of adaptor 2 corresponding to PCR primer 2. Clear boxes represent the inner part of the adaptors and corresponding nested PCR primers adapted from Diatchenko et al., 1996.

Two hybridizations are then performed. In the first, an excess of driver is added to each of the samples of the tester. The samples are then heat denatured and allowed to anneal. During the hybridization, single stranded tester molecules which have a complementary strand in the driver mostly form tester-driver heterohybrids (c) and are removed from the single-stranded (ss)-fraction (a).

Tester cDNAs can also be removed from the ss-pool by reannealing of complementary ss tester cDNAs forming ds tester homohybrids (b). This process is faster for highly abundant cDNAs in the tester cDNA as compared to low abundant cDNAs. Therefore the hybridization also leads to a normalization of the ss cDNA tester fraction (a) e.g. concentrations of high and low abundance cDNAs become roughly equal (Gurskaya et al., 1996).

In the second hybridization, the samples from the first hybridization are mixed together without denaturing in the presence of freshly denatured driver cDNA. Only the normalized and subtracted ss tester cDNAs are able to reassociate and form new type (e) molecules. The newly formed hybrids (e) have an important feature that distinguishes them from hybrids (b) and (c) formed during the first and second hybridizations. This feature is that they are double-stranded (ds) tester molecules with different ends, which correspond to adaptor sequences 1 and 2. These molecules with the different adaptors 1 and 2 or either end are the only ones which will be efficiently, e.g. exponentially, amplified in the subsequent PCR reaction. An extension reaction is performed to fill in the sticky ends of the molecules for primer annealing before the initiation of the PCR procedure.

In all PCR cycles, exponential amplification can only occur with type (e) molecules which have different sequences at their ends. Type (b) molecules contain long inverted repeats on the ends and form stable 'panhandle-like' structures after each denaturation-annealing PCR step. The resulting 'panhandle-like' structure cannot serve as a template for exponential PCR, because intramolecular annealing of long adaptor sequences is both highly favoured and more stable than intermolecular annealing of the much shorter PCR primers (Siebert et al., 1995). This effect is called 'suppression' PCR. Type (a) and (d) molecules do not contain primer binding sites and so can not be amplified. Type (c) molecules can be amplified only at a linear rate as they contain annealing sequence for only one of the primers. A secondary PCR amplification is then performed using nested primers to reduce background and enrich for differentially expressed sequences.

2.2.2.2 Generation of the subtractive library from 5L cells (-/+ TCDD)

Poly(A)$^+$ RNA was first prepared from non treated and 4 hours TCDD treated 5L cells. Northern hybridizations were done to confirm the presence of known TCDD-induced transcripts such as cytochrome P450 1A1 *(CYP450 1A1)* and glutathione-S-transferase Ya *(GST-Ya)* in the mRNA prepared from TCDD-treated cells. Both *CYP450 1A1* and *GST-Ya* were induced after 4 hours of TCDD treatment as shown in figure 19. cDNA was synthesized from poly(A)$^+$ RNA of non-treated (driver) and TCDD-treated (tester) cells. Subtractive hybridization was then performed using the PCR-select™ cDNA subtraction method to enrich for differentially expressed sequences in the tester cDNA population. The different steps in the screening system subsequent to the SSH are depicted in form of a flow chart in figure 20. Prior to screening of the subtractive library it is important to monitor the efficiency of subtraction.

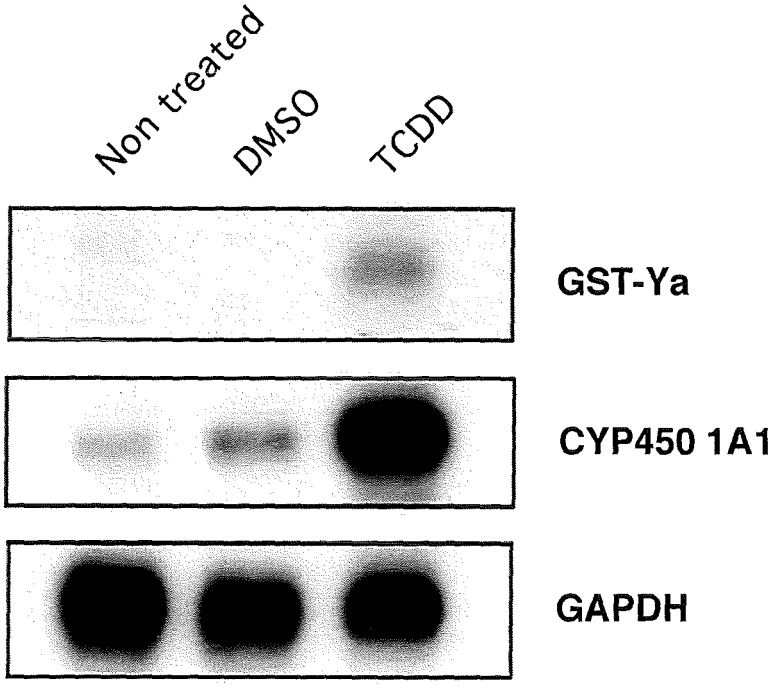

Figure 19. Quality control of mRNA used for subtractive hybridization.
Induction of two known genes was confirmed after TCDD treatment in the mRNA used for subtractive hybridization. Northern blot analysis was performed using 2 µg poly(A)+ RNA for CYP450 1A1 and GST-Ya mRNAs after 4 h treatment with 0.1% DMSO solvent or 1 nM TCDD. Loading of RNA was controlled by rehybridization with a GAPDH probe.

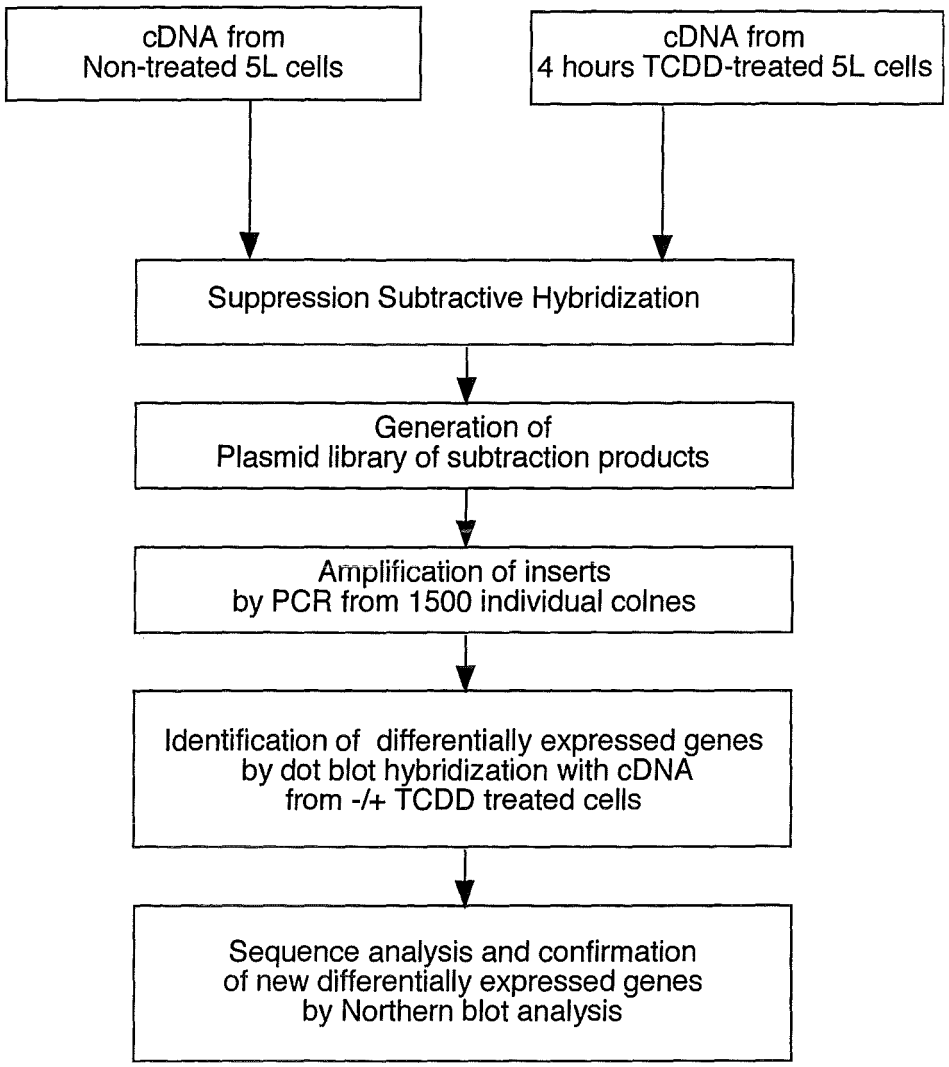

Figure 20. Flow diagram of the screen to find TCDD induced genes.

2.2.2.3 Efficiency of subtraction

The subtraction efficiency is determined by monitoring depletion of transcripts common to both driver and tester populations and enrichment of sequences specific to 'tester' population after subtraction. Southern blot analysis of subtracted and unsubtracted tester cDNA samples was done using one abundant non-differentially expressed gene, glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* and two known TCDD-induced genes *CYP450 1A1* and *GST-Ya*. *GAPDH* was almost completely removed after subtraction indicating the efficient removal of non-differentially expressed genes (figure 21). The cDNAs of *CYP450 1A1* and *GST-Ya* two known tester specific genes were enriched in the subtracted cDNA population (figure 21). This is an indication that the subtraction

had proceeded efficiently. The subtracted secondary PCR products were cloned into a plasmid vector. The resulting clones were analysed by the following method to identify the differentially expressed clones.

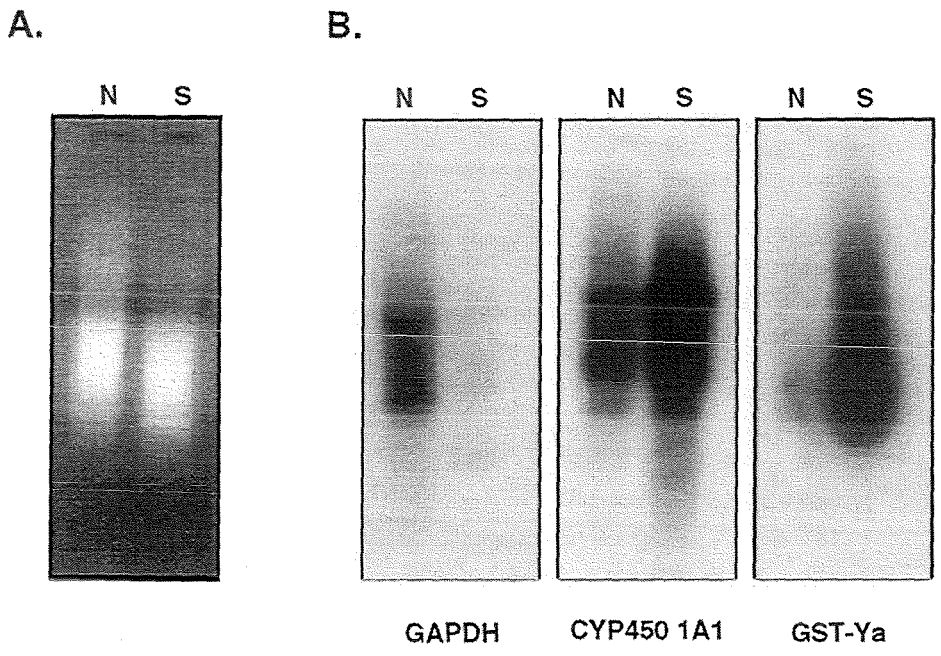

Figure 21. Monitoring of efficiency of subtraction. PCR amplified non-subtracted tester (N) and subtracted tester (S) cDNAs were separated in triplicates (approximately 500 ng per lane) on a 1.5% TAE agarose gel and transferred to a Hybond-N+ membrane under alkaline conditions. A photograph of the gel stained with ethidium bromide before transfer is shown in (**A**). (**B**) The membranes were probed with radioactive cDNAs of GAPDH, CYP4501A1 and GST-Ya

2.2.2.4 Identification of differentially expressed genes in the subtractive library

To identify the differentially expressed clones in the subtractive library, individual clones were picked and the inserts were amplified by colony PCRs. DNA is denatured with alkali treatment and duplicate set of dot blots were prepared in 96 well-format. These dot blots were then hybridized with radioactively labelled ds cDNA derived from either non-treated or 4 hours TCDD-treated cells. An example is shown in figure 22. To control hybridization conditions, each set of dot blots had two dots (25 and 50 ng) each of non-differentially expressed *GAPDH* and differentially expressed *CYP450 1A1*. Equal intensities of signals obtained with *GAPDH* indicated similar hybridization conditions.

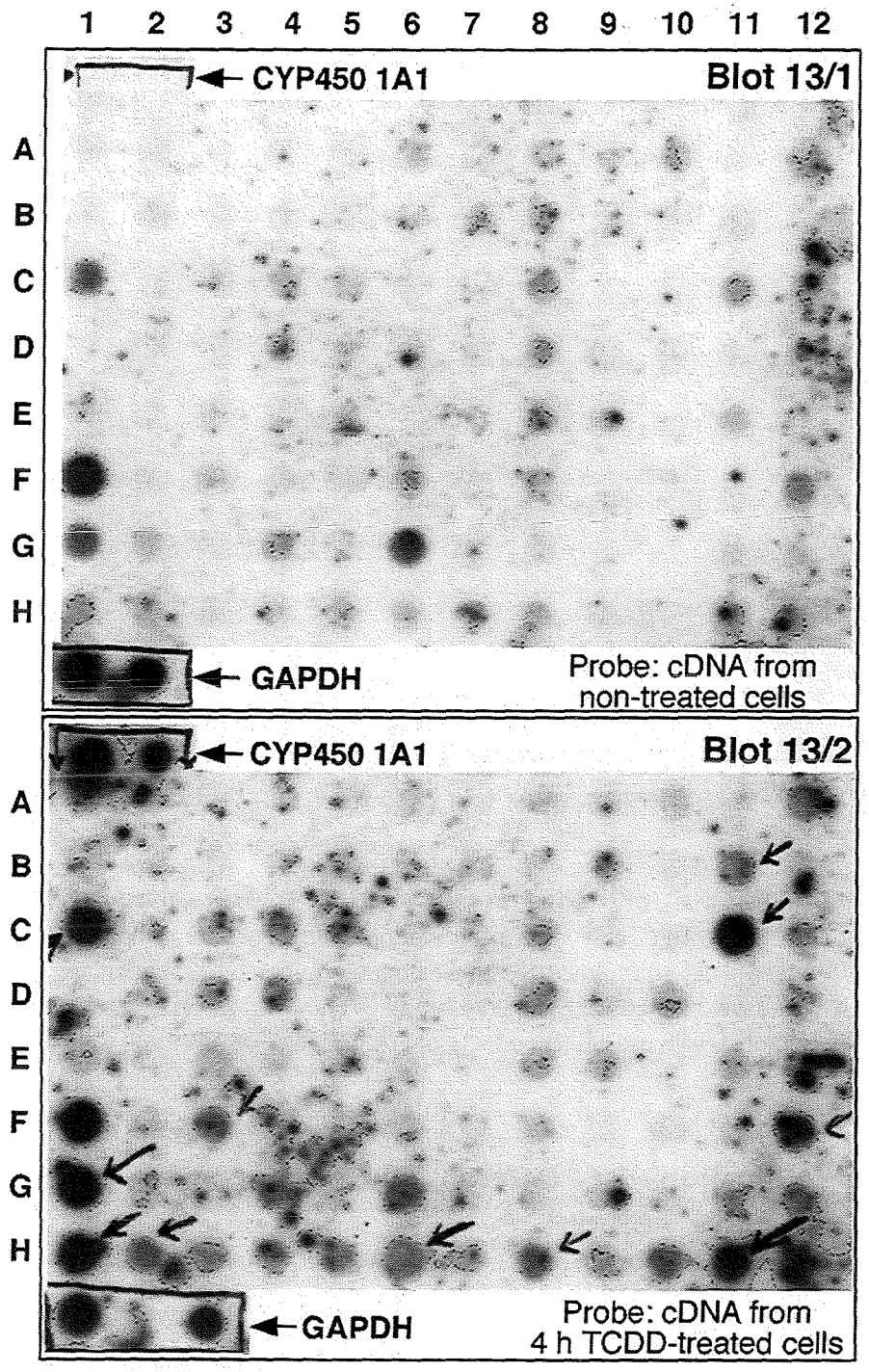

Figure 22. Differential screening of the subtractive library. Subtraction products were amplified individually by colony PCRs and duplicate dot blots were prepared. Each set of blots had two dots of cDNA (25 and 50 ng) each of non-differentially expressed GAPDH and differentially expressed CYP450 1A1 to control hybridisation. To identify cDNAs that represent TCDD-induced genes the DNA dot blots were hybridised with Rsa I digested radio-labelled cDNA from non-treated (Blot 13/1) or 4 h TCDD-treated (Blot 13/2) cells. Arrows indicate the clones corresponding to mRNAs that are differentially expressed in 5L cells after 4 h TCDD treatment. Equal intensities of signals were obtained with GAPDH indicating similar hybridisation conditions. CYP450 1A1 gave a signal only in the blot 13/2, probed with cDNA from 4 h TCDD-treated cells and served as a positive control for differentially expressed genes.

CYP450 1A1 gave a signal only in dot blot 13/2, probed with cDNA from 4 hours TCDD treated cells and acted as a positive control for differentially expressed genes. The clones which preferentially hybridized with radiolabelled cDNA from 4 hours TCDD treated cells represent potential cDNA clones induced by TCDD (figure 22, for example compare the dots at position H1). The clones that gave equal hybridization signals with both probes represent clones that were not differentially expressed (for example compare the dots at position F1). The hybridization signals obtained with different clones varied considerably which indicate the relative abundance of those genes in the cDNA used as radiolabelled probe. The clones which did not give any hybridization signal may represent low abundant mRNAs which in consequence are poorly represented in the radiolabelled cDNA probe. Though possibly differentially expressed, dot blot hybridizations are not sensitive enough to identify this class of low abundant cDNAs.

By this approach, 1500 clones were analysed and 34 of them hybridized preferentially with radiolabelled cDNA from 4 hours TCDD-treated cells.

2.2.2.5 Sequencing and confirmation of TCDD-inducibility of clones by Northern blot analysis.

To know the identity of the clones that hybridized preferentially with radiolabelled cDNA from TCDD-treated cells, plasmid DNA was isolated from the initial bacterial cultures and inserts were sequenced. The sequence analysis revealed that some of the genes that gave a differential signal on the dot blots were already known to be induced by TCDD. The isolation of such genes from a subtractive library indicated that the experimental approach to isolate TCDD-induced genes had been successful.

Besides the known TCDD-induced genes (group I in table II), there are 3 known genes (group II) and 8 unknown genes (group III). It is possible that some of the clones classified as unknown genes are from hitherto not sequenced or not published regions of known genes.

From the known directly TCDD-inducible genes I found cytochrome *P450 1A1*, cytochrome *P450 1B1*, aldehyde dehydrogenase, glutathione-S-transferase Ya, NAD(P)H:quinone reductase and UDP-glucuronosyl transferase (Hankinson, 1995). The genes given in group II are known but not reported to be induced by TCDD such as the B-cell translocation gene 1 (BTG1) (Rouault et al., 1992) lost on transformation 1 (LOT1) (Abdollahi et al., 1997) and transglutami-

nase (Gentile et al., 1991). Novel sequences without any data base matches are given in group III. Differential expression of clones in groups II and III was confirmed by Northern blot analysis (figure 23). The inserts of these clones were amplified by colony PCR, digested with Rsa I to remove adaptor sequences, and gel purified before they were radioactively labelled. Inserts were hybridized against poly(A)$^+$ RNA derived from non treated, 4 or 24 hours TCDD treated 5L cells. The Northern blots of 11 TCDD-induced clones (groups II and III) are shown in figure 23.

TABLE II

Summary of sequencing data of TCDD-induced clones

	Identity of the clone	Number of times picked
GROUP I		
1.	Cytochrome P450 1A1	4
2.	Cytochrome P450 1B1	1
3.	Aldehyde dehydrogenase	13
4.	Glutathione-S-transferase Ya	1
5.	NAD(P)H : quinone reductase	1
6.	UDP-glucuronosyl transferase	2
GROUP II		
7.	B-cell translocation gene 1	1
8.	Lost on transformation 1	1
9.	Transglutaminase	1
GROUP III		
10.	clone 106	1
11.	clone 1514	2
12.	clone 1525	1
13.	clone 1531	1
14.	clone 361	1
15.	clone 750	1
16.	clone 735	1
17.	clone 1396	1

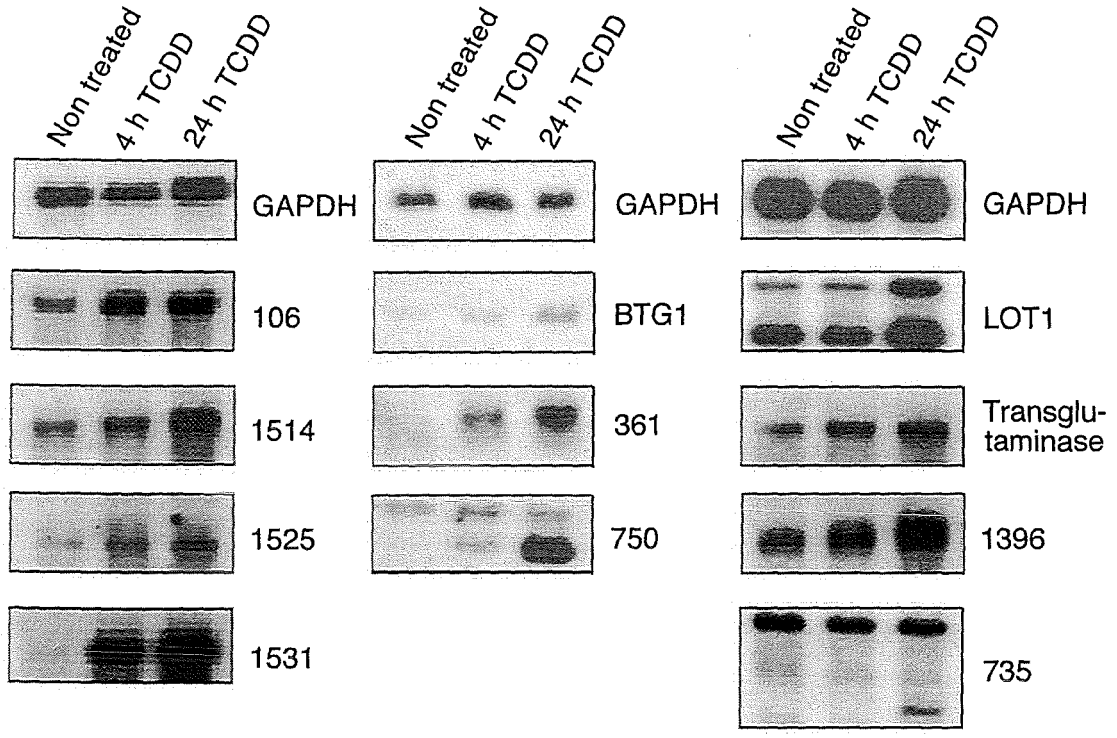

Figure 23. Northern blot analysis of TCDD-induced clones identified by subtractive hybridisation. 5 µg of poly(A)+ RNA from non treated (control), 4 and 24 h TCDD-treated 5L cells was analysed in Northern blot hybridisation using radio-labelled cDNA from individual clones. Equal loading of RNA was confirmed by rehybridisation of the blots with a GAPDH probe. The numbers refers to the clones depicted in Table II. Lot1 is lost on transformation (Abdollahi et al., 1997). BTG1 is B cell translocation gene 1 (Rouault et al., 1992).

The inducibility of the clones differed from one to another. There are clones which are induced >10 fold (clones 1531, 750 and 735) and others which are induced <4 fold (*BTG1, LOT1,* transglutaminase, clones 106, 1396 and 1525). This indicates that the experimental approach followed is also suitable to get clones of genes which are not heavily (<4 fold) regulated. The majority of the clones are clearly induced already after 4 hours of TCDD treatment and stay induced after 24 hours of treatment. There are clones, however which are clearly induced only after 24 hours of TCDD treatment (transglutaminase and 1396). Some clones gave two distinct bands on the Northern blot, only one of which is induced by TCDD. These clones might represent differentially spliced genes or two closely related genes, out of which only one is induced by TCDD (clones 735 and 750). Alternatively the double bands on Northern blots might also be a result of an artefact in generation of the subtractive library if inserts from two genes

would have been ligated into one clone out of which only one is regulated by TCDD.

2.3 Analysis of new TCDD-induced clones obtained through subtractive hybridization.

Subtractive hybridization was done to find TCDD-induced genes which led to the identification of eleven sequences whose expression was increased in 5L cells after TCDD treatment. Three of the sequences have homology to the known genes which are B-cell Translocation Gene 1 (BTG1), Transglutaminase and `Lost On Transformation´ 1 (LOT1). No data base matches were found for the remaining 8 sequences.

BTG1 is reported to negatively regulate cell cycle proliferation (Rouault et al., 1992). Transglutaminases are calcium-dependent enzymes that catalyse the cross-linking of proteins by promoting the formation of isopeptide bonds between protein-bound glutamine and lysine residues (Gentile et al., 1991). LOT1 is a novel gene recently cloned based on its loss of expression in malignantly transformed cell lines (Abdollahi et al., 1997).

The role of BTG1 was tested in TCDD-induced delay of 5L cell proliferation. A full length clone of BTG1 was obtained by RT-PCR using primers which were based on the published sequence information. BTG1 was overexpressed in sense or antisense orientations together with GFP by transient transfections to test its effect on 5L cell proliferation. Analysis was done as described in section 2.1.2. BTG1 overexpression had no effect on the proliferation of cells in the absence of TCDD. Antisense expression of BTG1 also did not affect TCDD-induced delay of 5L cell proliferation (data not shown). Hence BTG1 does not seem to play a role in TCDD-induced delay of 5L cell proliferation.

The significance of Lot1 and transglutaminase induction by TCDD is difficult to test in the absence of any functional assay. Every TCDD-induced gene may not be functionally linked to cell cycle effects of TCDD in 5L cells.

In addition to the three known genes, 8 novel TCDD-induced cDNA fragments were identified by subtractive hybridization. The starting point in the analysis of novel differentially expressed genes is to isolate longer/full length fragments of their cDNAs. Analysis will indicate if the sequence is part of the untranslated region of a known gene or truely a novel gene, meaning that no significant homology exists with the known genes reported in the data bases.

2.3.1 Cloning of longer fragments of a novel TCDD-induced gene

Since it is not possible to analyse all 8 novel TCDD-induced sequences due to time limitation, only clone 106 was picked for further analysis.

Longer fragments of clone 106 were obtained by rapid amplification of cDNA ends (RACE). An adaptor ligated double stranded cDNA (Marathon-Ready cDNA™ from Clontech Laboratories, Inc., USA) was used as a template for RACE. Two gene-specific primers in different orientations and one adaptor specific primer are required for performing 5' and 3'-RACE PCRs. Schematic representation of this procedure is shown in figure 24. This procedure was successfully used to isolate full length genes (Frohman et al., 1988 and Selmin et al., 1996).

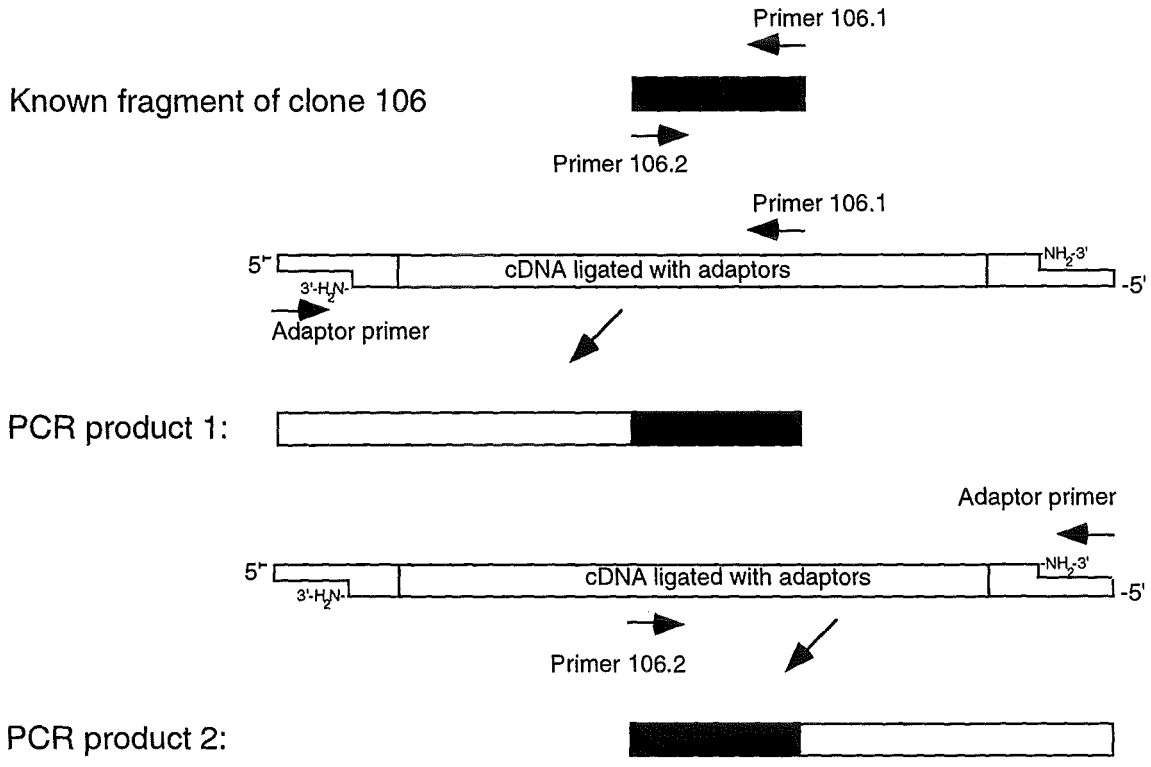

Figure 24. Amplification of longer fragments of a TCDD-induced novel sequence. Gene specific primers (106.1, reverse; 106.2, forward) were designed based on the known sequence of clone 106 (filled box). Double stranded cDNA ligated with adaptors (Clontech Laboratories, Inc., USA) was used as a template using the adaptor primer in combination with one of the two gene specific primers in separate PCR reactions, respectively. The 3' end of the adaptor is blocked with an amino group which prevents extension of the 3' end and formation of a binding site for the adaptor primer. The binding site for the adaptor primer is generated only by the extension of the gene specific primers.

Primers were designed based on the known sequence of clone 106 and RACE analysis for both 5' and 3'-ends was carried out. Two PCR products of 3.0 kb and 2.5 kb were obtained by 5' and 3' RACE. The PCR products were cloned into a vector and partial sequence was obtained from each end of the PCR products. Both the PCR products contained the sequence of clone 106, indicating that they represent the longer fragments of clone 106.

2.3.2 N-Myristoyltransferase 2 (NMT-2) is the novel TCDD-induced gene

Sequence analysis indicated that the 5' RACE product of clone 106 has 95% homology to the 5' part of the recently cloned mouse N-Myristoyltransferase 2 (NMT-2) cDNA (Giang and Cravatt, 1998). No data base homology was found to the 3' RACE product. This indicates that clone 106 is part of untranslated region of the NMT-2 gene which was not a part of the initially isolated and published clone.

N-Myristoyltransferase (NMT) is an enzyme catalyzing protein N-myristoylation which is the co-translational linkage of myristic acid, to the N-terminal glycine residues of some eukaryotic cellular proteins (Johnson et al., 1994). Examples of NMT substrates include protein kinases such as the catalytic subunit of cAMP-dependent protein kinase (PKA) and $p60^{Src}$. N-myristoylation was reported to play a causal role in transformation of cells by $p60^{v\text{-}Src}$ (Kamps et al., 1986). NMT protein and its activity has been shown to increase in colon tumors (Raju et al., 1997).

2.3.3 NMT-2 mRNA induction by TCDD does not require new protein synthesis

To test whether NMT-2 is a direct target gene of the AhR, TCDD-inducubility of NMT-2 mRNA was tested in the presence of the protein synthesis inhibitor cycloheximide (figure 25). TCDD induced NMT-2 mRNA both in the absence and presence of cycloheximide. Thus, ongoing or new protein synthesis is not required for induction of NMT-2 mRNA by TCDD indicating that NMT-2 is directly regulated and a new target gene of the AhR.

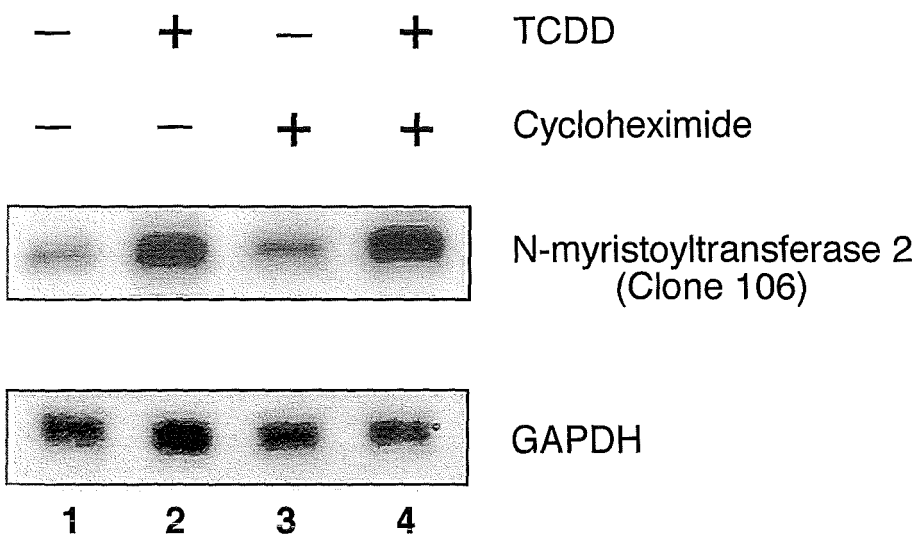

Figure 25. N-myristoyltrasferase 2 (NMT-2) mRNA induction by TCDD does not require new protein synthesis. Induction of NMT-2 (clone 106) mRNA was tested after 4 h exposure to 1 nM TCDD or 0.1% DMSO solvent with or without 30 min of pre-treatment with the translational inhibitor cycloheximide (20 µg/ml) by Northern blot analysis using 5 µg of poly(A+) RNA. As a loading control, the Northern blot was re-probed with GAPDH cDNA.

2.3.4 Inducibility of NMT-2 mRNA in livers of mice treated with TCDD

One possibility to form a hypothesis on the role of NMT-2 in mediating any of the effects of TCDD *in vivo* is to test its inducibility in a target organ of dioxin toxicity. TCDD is a potent promoter of hepatocarcinogenesis. So, the inducibility of NMT-2 mRNA in livers of TCDD-treated mice was tested. Semi-quantitative RT-PCR was done with total RNA from livers of TCDD-treated mice (liver samples were kindly provided by Prof. Michael Schwarz, Tuebingen), using gene specific primers to NMT-2 and GAPDH in a single tube. The RT-PCR products were analysed by Southern blot hybridization for NMT-2 and GAPDH (figure 26). The signals obtained with NMT-2 were normalized to the signals obtained with GAPDH. Analysis of the results indicated that NMT-2 mRNA was upregulated in livers of TCDD-treated mice (by 2.5 fold after 5 days and 4 fold after 7 days of treatment).

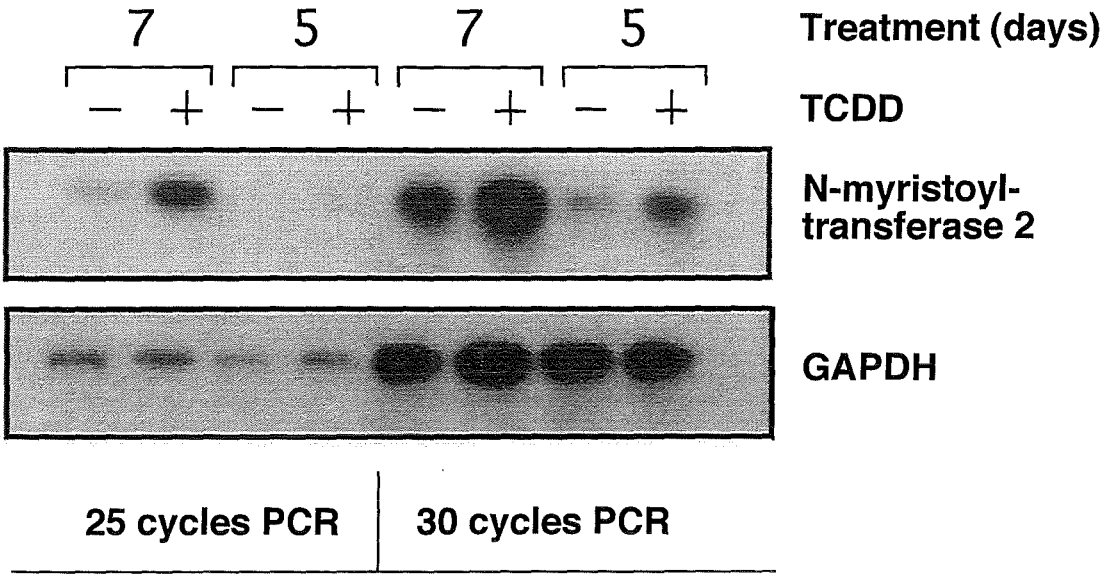

Figure 26. N-myristoyltransferase 2 (NMT-2) mRNA is induced in livers of TCDD treated mice. Total RNA was prepared from livers of mice 5 or 7 days after they had received a single injection of solvent (-) or TCDD (+; 1.4 µg/kg body weight). RT-PCR was done as described in methods and materials using primers specific to NMT-2 (106.1 and 106.2) and GAPDH (GP3 and GP5) in a single tube. Samples were collected after 25 or 30 cycles of PCR. PCR conditions were 94°C for 30 sec, 58°C for 45 sec and 72°C for 90 sec. PCR products were separated on a 1% TAE-agarose gel, denatured and transferred to a membrane under alkaline conditions. Hybridisations were done with radio-labelled NMT-2 or GAPDH cDNAs.

In summary, subtractive hybridization led to the identification of 34 TCDD-induced clones in 5L cells. Out of them, 22 were known TCDD-induced genes coding for drug metabolizing enzymes. The remaining 12 clones, represent 8 novel sequences and 3 known genes whose expression was previously not known to be induced by TCDD. Cloning of a longer fragment of a novel sequence has identified it as part of the N-Myristoyltransferase 2 gene which in addition to 5L cells is induced in livers of TCDD-treated mice.

3. Discussion

The AhR mediates toxic and carcinogenic effects of dioxins (Huff et al., 1994). Known AhR regulated genes mostly code for xenobiotica metabolizing enzymes but AhR regulated genes responsible for dioxin toxicity were largely unknown. The aim of this work was to identify target genes of AhR relevant for dioxin toxicity.

3.1 Choice of the model system

Choosing the right system for studying dioxin toxicity and searching for the AhR target genes is utmost important. At the beginning of this work, I had to choose between an *in vivo* or a cell culture model system. The advantages and disadvantages of these two systems are discussed below.

In vivo studies with TCDD-treated animals provide an opportunity to directly analyse the affected organs. However, mechanistic studies which involve gene transfer and gene inactivation are difficult and tedious to perform *in vivo*. Generating knock-out or transgenic mouse strains are feasible in principle, but these techniques can not be used for screening purposes, e.g. analysis of many site-directed mutations in a protein of interest.

The cell culture system which was available to me was 5L rat hepatoma cells. The 5L cells are genetically well defined with respect to the expression of AhR and the effects of TCDD. TCDD severely delays proliferation of 5L cells. This constitutes one of the very few toxic actions of TCDD in continuously growing cells in vitro. In the 5L cell system, AhR-deficient and TCDD-resistant variant subclones, the BP8 and the $BP8^{AhR+}$ cells are available (Göttlicher et al., 1990). The lack of AhR is the only defect which renders BP8 cells resistant to TCDD, since stable expression of AhR in these cells restores TCDD sensitivity completely (Weiß et al., 1996). 5L cell cultures are composed of a single cell type. They offer a possibility for analysing primary events in TCDD toxicity since the effects on proliferation are manifested within a few hours. The cell culture system allows the usage of transcriptonal/translational inhibitors which help in determining the direct effects of dioxins on gene expression. Large sets of experimental points can be collected in cell culture for example in studying dose-response relationships. Furthermore, gene transfer experiments can be

performed easily in cell culture and low amounts of dioxins are required compared to what is needed for *in vivo* experiments.

Keeping the several advantages in mind I had decided for 5L cell culture system for studying dioxin toxicity. However, a general concern in using cell culture systems is to what extent they do reflect a cell type and a response pathway being relevant in the whole organism. So the findings in *in vitro* systems have to be validated *in vivo*. This has been done successfully in this study and is discussed in section 3.4.

3.2 A positively induced genetic programme for TCDD-induced cell cycle delay

Having chosen 5L cell system for studying TCDD toxicity, I wanted to determine how AhR mediates the delay in proliferation of these cells. There is evidence in literature for diverse functions of AhR after TCDD treatment. The AhR regulates the expression of a number of genes involved in drug metabolism (reviewed in Schmidt and Bradfield, 1996). The receptor is reported recently to interact with cell cycle regulatory proteins like Rb and cyclin-CDK complexes (Greenlee-personal communication; Ge and Elferink 1998). A reduction in epidermal growth factor (EGF) binding in liver plasma membranes of TCDD-treated rats was shown to be due to a decline in the number of EGF receptors (Hudson et al., 1985; Lin et al., 1991 and Madhukar et al., 1984). Increased phosphorylation and activity of protein-tyrosine kinases such as $p60^{c\text{-}Src}$ in thymus of TCDD-treated mouse was reported (Enan and Matsamura, 1995). It is not known how AhR mediates such diverse effects. There is, however, evidence from studies of steroid hormone receptors that one and the same receptor can serve qualitatively quite distinct functions which are described below.

Transcription factors are classically described as sequence-specific DNA-binding proteins which activate transcription of genes. In addition to this classical mode of action at least two more functions of steroid receptors have been discovered in recent years (Göttlicher et al., 1998). Firstly, steroid receptors have been shown to modulate the activity of other transcription factors apparently without binding to DNA themselves. Rather, this type of so called 'transcriptional cross-talk' is likely to be based on protein-protein interactions. A typical example of this cross-talk is the mutual interference of the glucocorticoid receptor and the

AP-1 family of transcription factors (Jonat et al., 1990 and Schuele and Evans, 1991). Most frequently negative interference of both factors with each other's activity has been observed but also positive 'Cross-talk' is possible under certain conditions (Diamond et al., 1990; Shemshedini et al., 1991 and Teurich and Angel, 1995). Secondly, transcription factors (e.g. androgen and estrogen receptors) are reported to induce signal transduction (MAPK/SAPK signalling) cascades. These so called 'non-genomic actions' are independent of the receptor acting on chromatin in the cell nucleus (Migliaccio et al., 1996 and Peterziel, 1998)

Different functions of the steroid receptors could be dissociated with the use of mutants which can perform one function but not the other. Specific mutations in the DNA binding domain of glucocorticoid receptor for example, affect its ability to bind DNA and transactivate genes but do not affect its ability to repress AP-1 transcription factors (Heck et al., 1994). Similarly, mutants of androgen receptor defective in the nuclear localisation can not directly transactivate genes but still can induce signal transduction cascades (Peterziel, 1998). Knowing the different actions of steroid receptors, I wanted to determine whether AhR positively induce a genetic programme for cell cycle delay and to obtain circumstantial evidence how AhR could induces such a delay. This was done by mutational analysis of the AhR in receptor deficient BP8 cells.

Expression of wild type but not of any of the mutant forms of AhR defective in DNA binding or transactivation reconstituted TCDD-induced cell cycle delay in BP8 cells. Thus, both DNA binding and transactivation functions of the receptor appear to be essential for mediating cell cycle effects of TCDD. This suggested that transcriptional activation of genes by AhR is required for mediating TCDD-induced cell cycle delay.

One could argue that the mutants of AhR used for reconstitution of cell cycle delay are also defective in other functions of the receptor. It is, however, unlikely that all four mutants of the AhR tested (one of them having a single amino acid exchange in the DNA binding domain) are deficient with respect to a specific but hitherto unknown function of the receptor distinct from activation of target genes upon binding to DNA.

There is further support for the requirement of transcription in TCDD-induced cell cycle delay. TCDD inhibited G1-phase progression prior to

hyperphosphorylation of the retinoblastoma protein (Rb) in 5L cells synchronously released from a low serum-induced cell cycle arrest. This effect of TCDD was antagonised by the transcriptional inhibitor actinomycin D, although actinomycin D alone during the same short time period did not inhibit Rb hyperphosphorylation (Carsten Weiß - personal communication). This suggests that ongoing and presumably induced gene transcription (likely but not necessarily by AhR) is required for cell cycle effects of TCDD in 5L cells. This requirement for transcription together with the results of the mutational analysis, argues for AhR-dependent transcription in TCDD-induced cell cycle delay. Having this as the basis, I searched for AhR regulated genes responsible for cell cycle delay in 5L cells.

3.3 Search for target genes of AhR

The search for AhR target genes was done by two different ways. The first was an 'educated guess' approach analysing the known cell cycle inhibitory proteins. The second was an unbiased search for TCDD-induced genes.

3.3.1 Transcriptional induction of $p27^{Kip1}$ by AhR inhibits 5L cell proliferation

The $p27^{Kip1}$ cell cycle inhibitor was found to be upregulated upon TCDD treatment in 5L cells. This induction is specific because the levels of other inhibitors did not change substantially or were not detected (Martin Göttlicher - personal communication). AhR mediates $p27^{Kip1}$ induction, since TCDD-dependent $p27^{Kip1}$-upregulation was not found in the receptor deficient BP8 cells. Furthermore, the induction was restored by stable expression of AhR in BP8 cells (data not shown).

Induction of $p27^{Kip1}$ occurs through the direct induction of Kip1 mRNA by AhR as the translational inhibitor cycloheximide did not prevent Kip1 mRNA induction. This is a novel mode of Kip1 regulation distinct from previously described posttranscriptional mechanisms. In every case reported so far $p27^{Kip1}$ induction occurred through increased stability of the protein (Pagano et al., 1995) and/or increased rate of translation (Hengst and Reed, 1996 and Millard et al., 1997). Posttranscriptional regulation of Kip1 in 5L cells upon TCDD treatment is

unlikely as the half lives of mRNA and protein did not differ in TCDD treated and control cells.

Induction of $p27^{Kip1}$ is the cause for TCDD-induced cell cycle delay. Genetic evidence for this was generated by the expression of antisense Kip1 RNA which impaired the accumulation of TCDD-induced $p27^{Kip1}$ and reversed the cell cycle effects of TCDD in 5L cells.

3.3.1.1 How is Kip1 regulated?

The AhR regulates transcription of the xenobiotic metabolizing enzymes as a heterodimeric complex with Arnt by binding to XRE sequence(s) (Schmidt and Bradfield, 1996). However, the regulation of Kip1 seems to be different from that of xenobiotic metabolizing enzymes. The human and murine promoters of Kip1 have been cloned and do not contain a consensus XRE (Kwon et al., 1996 and Minami et al., 1997). Induction of a reporter gene containing the luciferase cDNA under control of the murine Kip1 promoter by TCDD suggests that the cloned promoter has the necessary elements required for conferring inducibility (Carsten Weiß - personal communication).

The dose-response curves for induction of mRNAs for Kip1 and the classical XRE-regulated gene *CYP450 1A1* were different. The half-maximal induction of Kip1 and *CYP450 1A1* mRNAs was observed at TCDD concentrations of 36 and 14 pM respectively. These observations could mean that AhR regulates Kip1 differently from xenobiotic metabolizing enzymes. Such putative other modes of AhR-function could be independent of Arnt and evidence for that is discussed below.

Arnt and DNA binding defective mutant Arnt were overexpressed in 5L cells to determine the requirement of Arnt in TCDD-induced cell cycle delay. If activation of genes by the AhR-Arnt complex was required, overexpression of Arnt was expected to synergise with AhR activation by TCDD and mutant Arnt should act dominant negatively. However, the observed effects of Arnt and mutant Arnt were opposite to what was expected. Overexpression of full length Arnt led to the reversal of TCDD-induced cell cycle delay, whereas the DNA binding defective mutant Arnt had no effect. This suggests that the mechanism of

TCDD-induced cell cycle delay is unrelated to transcriptional regulation by the AhR-Arnt complex.

There are two possible ways to explain how Arnt can antagonise AhR mediated cell cycle delay. Arnt is shown to form homodimers and bind to E-box sequence *in vitro* in the absence of AhR (Swanson et al., 1995). Arnt is also shown to heterodimerize with other factors (e.g. hypoxia inducible factor 1α) and regulate transcription of genes independent of AhR or dioxin (Wang and Semenza, 1995).

Thus, the ligand-AhR may sequester Arnt into complexes with AhR and limit the amounts of Arnt available for other functions which are required for cell cycle progression. It is unlikely that this is the mechanism for TCDD-induced cell cycle delay because the mutants of AhR which have intact dimerization capacity with Arnt did not have any effect on cell cycle in the AhR deficient BP8 cells (refer section 3.2).

The second possibility is that AhR mediates cell cycle effects of TCDD without a partner protein as a homodimer or together with partner protein(s) distinct from Arnt. With Arnt overexpression, the AhR mediated cell cycle effects are reversed possibly owing to the sequestration of limiting amounts of AhR or a putative partner protein of AhR by Arnt.

To solve this question requires to understand in detail how AhR regulates the Kip1 promoter, e.g. delineation of the responsive elements in the promoter and characterisation of the protein complexes binding to these elements.

3.3.2 Analysis of TCDD-induced genetic programme by suppression subtractive hybridization (SSH)

3.3.2.1 Choice of the method

A number of techniques are currently available such as differential display (Liang and Pardee, 1992), representational difference analysis (Hubank and Schatz, 1994 and Lisitsyn et al, 1993), serial analysis of gene expression (Velculescu et al., 1995) among others to monitor changes in gene expression. Despite the fact that many differentially expressed genes have been cloned by

these methods, all have one draw back or the other. For example these methods are not suitable for the identification of cDNAs of rare transcripts which are differentially expressed. The reason is that the original disproportion in the concentration of high- and low-abundant fractions is maintained in most of the procedures and often only the cDNAs of most abundant transcripts are isolated. However, the products of weakly expressed genes are also of interest, as they could play a role in the initiation and control of cellular processes. Recently, a new PCR-based cDNA subtraction method termed as suppression subtractive hybridization (SSH) has been described to obtain differentially expressed genes (Diatchenko et al., 1996). This is an ideal system for subtractive cloning as it equalizes representation of differentially expressed genes irrespective of their initial relative abundance. The high efficiency of this system was demonstrated by isolation of rare transcripts (Gurskaya et al., 1996; von Stein et al., 1997).

3.3.2.2 Analysis of the results of SSH

SSH was performed to identify TCDD-induced genes in 5L cells. Amongst the identified 34 differentially expressed genes, 22 were already known to be induced by TCDD. This indicated that the experimental approach to isolate TCDD-induced genes had been successful. Furthermore, the identification of genes which are induced <4 fold indicate that the experimental approach followed here is also suitable to identify genes whose expression is not altered drastically.

Besides the known TCDD-induced genes, there are 3 known genes (B-cell translocation gene 1, transglutaminase and lost on transformation 1) which were not reported to be induced by TCDD and 8 novel sequences without any data base matches.

3.3.2.3 Completelyness of the identified genetic programme

It is appropriate to ask whether all the differentially expressed genes present in the subtractive library have been identified. Remarkably, the expected gene for the TCDD effect on the cell cycle, Kip1, was not at all picked. Though not picked from the library, it had been present after completion of the subtraction reaction. Thus, the failure to isolate Kip1 by the SSH method was a problem of

too few colonies being analysed, rather than the SSH reaction to generate the differential library. The notion that 1500 analysed clones were too few for a saturating analysis was also supported by the finding that many genes were only picked once (refer Table II). Only two genes were picked multiple times (*CYP450 1A1* (4 times) and aldehyde dehydrogenase (13 times)). The reason why these genes were isolated particularly efficiently is not known. One has to conclude that the number of isolated clones did not suffice to cover the complete set of AhR-dependently induced genes. Amongst the expected genes to be found there was, however, only one gene out of 7 is missing. It is fair to assume that also amongst the novel genes the percentage of missed genes is similarly low.

3.3.2.4 N-Myristoyltransferase 2 is a novel TCDD-induced gene

One of the novel sequences induced by TCDD was chosen for further analysis. Longer fragments of that sequence were isolated by a PCR based RACE (rapid amplification of cDNA ends) approach. Sequence analysis has identified it as the gene coding for N-Myristoyltransferase 2 (NMT-2). NMTs are enzymes catalysing protein myristoylation which is the co-translational linkage of myristic acid to the N-terminal glycine residues at position 2 of some of eukaryotic cellular proteins. Human and mouse cDNAs for two distinct NMTs termed as NMT-1 and NMT-2 have been cloned. The specific role of each NMT in protein myristoylation is not yet studied (Giang and Cravatt, 1998). Furthermore, nothing is known about the transcriptional regulation of NMTs. Thus, TCDD is the first substance known to induce at least one NMT transcriptionally.

3.4 Relevance of Kip1 and NMT-2 induction by TCDD in vivo

As discussed in section 3.1, the findings in cell culture systems have to validated *in vivo.* The inducibility of Kip1 and NMT-2 by TCDD was tested and found to be induced *in vivo* or in a primary system very close to *in vivo.* For Kip1, the relevance of induction in mediating TCDD toxicity was also tested.

3.4.1 Kip1 is causally linked to TCDD-induced inhibition of thymocytes in organ culture of fetal thymus glands

Induction of thymic aplasia by dioxins *in vivo* is reproducible in organ culture of thymus glands (FTOC) (Lai et al., 1997). FTOC was used to test if dioxins operated through induction of Kip1 in a primary system, as had been shown for immortalised 5L cells. TCDD induced Kip1 protein and decreased thymocyte proliferation in FTOC (Martin Göttlicher - personal communication). The causal role of Kip1 in TCDD effects on the fetal thymus was tested by establishing FTOCs from wild-type or Kip1-mutant litter-mate embryos. The targeted mutation in the Kip1 gene (Δ51 allele) impairs interaction with the cyclin/CDK complexes and hence does not permit inhibition of these kinase complexes (Kiyokawa et al., 1996). TCDD had only a minor effect in the FTOCs from Kip1 mutant mice as compared to FTOCs from wild type litter-mates (Martin Göttlicher, personal communication). This indicates that the major part of the TCDD response in FTOC is dependent on $p27^{Kip1}$. Thus the induction of Kip1 by TCDD plays a causal role in inhibition of cell proliferation not only in 5L cells but also in FTOC, which is more close to an *in vivo* situation. This proves that the use of a cell culture system is valid to identify genes (and signalling pathways) relevant for TCDD-toxicity.

Loss of Kip1 by targeted mutagenesis in mice leads to increased body weight and multiple organ hyperplasia including a hyperplastic thymus which is a phenotype largely opposite to the symptoms of dioxin poisoning (Fero et al., 1996; Kiyokawa et al., 1996 and Nakayama et al., 1996). The induction of Kip1 could thus explain some of the biological effects of dioxins such as thymic atrophy and sperm count reduction and reduced body weight gain in rodents, which are associated with reduced proliferation. Proof for this hypothesis has to be generated by the analysis of TCDD treated Kip1 knock-out mice.

3.4.2 NMT-2 is induced in livers of TCDD-treated mice

The first step in investigating the role of NMT-2 in mediating any of the effects of TCDD *in vivo* is to test its inducibility in a target organ of dioxin toxicity. Liver is a major organ of TCDD action as it is a carcinogen in the rodent liver (Huff et al., 1994). The inducibility of NMT-2 mRNA was tested and found to be increased in livers of mice after 5 or 7 days of TCDD treatment. Thus NMT-2 fulfills one essential requirement to be a candidate gene in mediating TCDD

toxicity e.g. it is induced in a target organ of dioxin toxicity. Whether NMT-2 really plays a role in TCDD induced toxicity is at the present stage of knowledge up to speculation. Published data on NMTs may help to form a hypothesis.

The substrates of NMT include protein kinases like $p60^{Src}$, cAMP-dependent protein kinase (PK-A) and phosphatases such as calcineurin B. Deletion or substitution of the N-terminal glycine at position 2 of N-myristoylated proteins by site-directed mutagenesis prevents their myristoylation, allowing comparison of the properties of the mutant, nonmyristoylated and wild type N-myristoylated proteins. For example glycine 2 to alanine mutagenesis of $p60^{v\text{-}Src}$ prevents its targeting to the plasma membrane and blocks its ability to transform cells (Kamps et al., 1986). In a different approach to prevent myristoylation by treatment of colonic cell lines with N-fatty acyl compounds a similar result was obtained. Inhibition of $p60^{c\text{-}Src}$ myristoylation depressed colony formation and cell proliferation (Shoji et al., 1990).

In colon tumors NMT protein and its activity has been shown to be increased compared to normal tissue (Magnuson et al., 1995 and Raju et al., 1997). Tyrosine kinase activities of $p60^{c\text{-}Src}$ and $p62^{c\text{-}Yes}$ protein kinases have also been shown to be elevated in primary colorectal adenocarcinoma (Bolen et al., 1987; Cartwright et al., 1993 and Park et al 1993). Although the analysis of the tumor can never prove the reason for its generation, it is tempting to speculate that an inappropriate increase in protein myristoylation plays an important role in pathogenesis. Since TCDD induces NMT-2 *in vivo*, it is tempting to speculate that this ectopic induction of NMT-2 plays a role in the induction of carcinomas by TCDD.

Protein myristoylation plays an important role also in other cellular and pathological processes, e.g. the assembly and viability of human immunodeficiency virus-1 (HIV-1) (Gottlinger et al., 1989). TCDD was shown to increase virus production in MT-4 human lymphoid cells infected with HIV-1 virus (Pokrovsky et al., 1991 and Tsyrlov and Pokrovsky, 1993). It is tempting to speculate that the increase of myristoylation activity after TCDD-treatment was the reason for the observed effects.

The following approaches will help to elucidate the role of NMT-2 induction in carcinogenicity of TCDD. The first step is to measure myristoyltransferase activity in livers of TCDD-treated mice. Since there are at

least two NMTs, it is important to test whether the increase in NMT-2 mRNA correlates with a corresponding increase in enzymatic activity. This will help to estimate whether NMT-2 plays a major role in myristoylation of proteins in liver. The next step is to test whether there is a correlation of NMT-2 expression and enhanced proliferation of cells (for example by immuno-histochemical examination) in livers of TCDD-treated animals. If the outcome of these experiments is still compatible with a role for NMT-2 in tumor promotion by dioxins, one could plan TCDD toxicity studies in NMT-2 knock-out mice once they are available. An alternative approach was to cotreat the animals with TCDD and specific inhibitors of NMT-2 enzymatic activity (if available) and determine whether the animals are resistant to TCDD-induced tumors.

3.5 Endogenous functions of AhR

The gene targeting technology to inactivate murine genes *in vivo* has been a powerful technique to elucidate the function of any gene product of choice. AhR knock-out mice have been generated by two different groups (Fernandez-Salguero et al., 1995 and Schmidt and Bradfield, 1996). The livers of these mice were reduced in size by 50 percent and showed bile duct fibrosis. In addition, the AhR knock-out mice generated by one group also had a phenotype in the immune system (Fernandez-Salguero et al., 1995). These mice showed decreased accumulation of lymphocytes in the spleen and lymph nodes. Thus the AhR is required for normal development of the liver and possibly the immune system. It is not known, however, how AhR becomes activated by an endogenous mechanism during development, e.g. whether there is an endogenous ligand for the receptor or whether AhR is activated by a ligand-independent mechanism. It is also not known which genes have to be regulated by AhR to ensure proper development of the liver and of the peripheral lymphatic organs.

Now the question is whether Kip1 or NMT-2, the new target genes of AhR, can explain the phenotype of the AhR knock-out mice. Lack of AhR in mice might lead to reduced expression of either or both of these genes during normal development. Comparison of the expression of Kip1 and NMT-2 in wild type and AhR knock-out mice could give a first hint about the involvement of these genes. Lack of Kip1 in mice leads to increase in body weight and also results in proportional increase in liver size. These mice also have increased accumulation of lymphocytes in spleen and lymph nodes (Fero et al., 1996; Kiyokawa et al.,

1996 and Nakayama et al., 1996). Thus, the phenotype of Kip1 knock-out mice does not correlate with that of AhR knock-out mice and it is unlikely that reduced/loss of expression of Kip1 explains the phenotype of AhR knock-out mice.

Whether AhR dependent expression of NMT-2 plays a role during development of liver and the immune system is not known. Certainly the development of thymocytes depends on the presence and most likely the myristoylation of Src-family tyrosine kinase Lck (Lowell and Soriano, 1996). Theoretically NMT-2 thus could be an AhR target gene which is required for normal thymic development, but too little is known at present to decide this question.

In summary, 12 (11 from SSH + Kip1) new target genes of AhR have been identified. Two of them, Kip1 and N-myristoyltransferase 2 (NMT-2) have been studied in detail. For both of them AhR constitutes the first pathway of conditional regulation on the transcriptional level. Kip1 is shown to play a causal role in inhibition of proliferation by TCDD in 5L cells and in fetal thymus glands. The induction of Kip1 could explain some of the biological effects of TCDD, such as thymic atrophy, which are associated with reduced cell proliferation. Since increase in myristoylation of proteins is implicated in carcinogenesis, it is tempting to speculate that the AhR-dependent induction of NMT-2 mediates carcinogenecity of dioxins.

The complexity of the genetic program regulated by AhR already in a single cell line suggests that there is no simple AhR regulated master gene for the physiological function of AhR and for the dioxin toxicity. The analysis of clearly defined cellular models and genetic profiling as performed in the present study provide the tools to understand the mechanisms of dioxin toxicity.

4. Materials and methods

4.1 Materials

All general chemicals were of the highest purity grade, the majority of which were supplied from *Merck*, Darmstadt, *Carl Roth GmbH & Co* Karlsruhe and *Sigma Chemie GmbH*, Deisenhofen. Radioactive chemicals were from Amersham Buchler (Braunschweig). Aqueous solutions were made with water purified (bi-dest) by a Milli-Q water purification system. A number of protocols and recipes for commonly used buffers were taken from the laboratory manual of Maniatis et al. (1989) and Current protocols in Molecular Biology (Ausubel et al., 1989) unless otherwise stated.

4.2 General methods

Cell lines

5L and BP8 cells: Rat hepatoma cells were supplied by Martin Göttlicher, Forschungszentrum, Karlsruhe and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Eggenstein).

Cell culture

All cells were maintained at 37°C in an incubator (Forma Scientific, Labotect GmbH, Göttingen) in 5% CO_2 and 95% air humidity. All cells were grown in culture dishes (Greiner Labortechnik, Frikenhausen) of varying sizes. The cells were allowed to grow until a confluency of 80-90% had been reached, whereupon the cells were trypsinized and either reseeded at a lower density or plated out for an experiment.

Trypsin treatment of cells was performed by removal of the culture medium from the cells, followed by one wash with 10 ml of Ca^{2+}/Mg^{2+} free PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 x 2 H_2O, 1.5 mM KH_2PO_4).
After removal of the PBS, cells were incubated with 1.0 ml of prewarmed 0.25% trypsin at 37°C until cells had become detached from the culture dish. The cells were resuspended in fresh medium and replated at the desired density.

Cell stocks were maintained in liquid nitrogen. Logarithmically growing cells were trypsinized and 10 ml of medium was added. Cells were collected by centrifugation at 250xg. The medium was removed and cells were resuspended in previously cooled 90% FBS and 10% DMSO (Fluka Chemie AG, Buchs, Switzerland) and transferred to cryo vials in 1 ml aliquots. The cells were kept on ice for an hour and transferred to -80°C for several hours before they were finally moved to liquid nitrogen. To re-propagate cells, the vials were removed from liquid nitrogen and thawed at 37°C. The cells were mixed with 10 ml of fresh medium and centrifuged at 250xg (to remove DMSO) and plated out on culture dishes with fresh culture medium.

Mini-prep plasmid preparation from bacteria

Individual colonies were picked from a LB agar plate and used to inoculate 3 ml of LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl), containing 100 µg/ml ampicillin. The inoculated bacteria were then incubated with shaking (220 rpm) overnight at 37°C until a stationary phase had been reached. 1.5 ml were removed and the bacteria were pelleted by centrifugation at 4000xg for 5 min.

(a) Using Phenol-Chloroform

Bacterial pellet was resuspended in residual 100 µl LB medium and 50 µl phenol were added. After vortexing, 5 min centrifugation at 10000xg the supernatant was mixed with equal amount of chloroform, again vortexed, centrifuged at 10000xg. Aqueous phase was taken to precipitate DNA with 2.5x volumes of ethanol at a final NaCl concentration of 0.1 M at RT for 30 minutes. DNA was collected by centrifugation at 10000xg, pellet was air dried and resuspended in 50 µl TE-buffer.

(b) Wizard Mini-prep kit (Promega)

Resuspension, lysis and neutralisation of the bacterial pellet was carried out according to the manufacturer's instructions using the provided buffers. The plasmid DNA in the retained supernatant was isolated using the supplier's DNA-binding resin. The resulting plasmid DNA was then eluted in 50 µl of bi-dest H_2O.

This method was primarily used for the production of sequencing grade plasmid DNA.

Large scale plasmid preparation from bacteria

Usually, a volume of 200-250 ml of LB (16 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) medium supplemented with the relevant antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37°C overnight until the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 4000xg for 10 min and the pellet resuspended in 10 ml of solution I (10 mM EDTA, 50 mM Tris-HCl pH 8.0 and 400 mg/ml of RNase A). Following 5-10 minutes of incubation at RT, the cells were lysed by the addition of 10 ml of solution II (200 mM NaOH and 1% SDS). Once the solution had taken an opaque appearance, the mixture was neutralized with 10 ml solution III (3 M potassium acetate pH 5.5). After additional 10-20 minutes on ice, the cell wall fragments and the bacterial chromosomal DNA were sedimented by centrifugation at 13000xg for 20 minutes at 4°C in a fixed angle rotor. The retained supernatant was then added directly to a pre-equilibrated Qiagen-tip 500 column (Qiagen Inc., Hilden) and the plasmid DNA was recovered according to the manufacturer's instructions using the supplied buffers. The purified DNA was precipitated using 0.7 volumes of isopropanol, washed twice in 70% ethanol before resuspending the DNA to a final concentration of 1-3 mg/ml in bi-dest H_2O and stored at -20°C.

Preparation of competent bacteria (E.Coli)

Chemical competent E.Coli (calcium chloride method)

A single colony of *E.coli* DH5α was taken to inoculate 5 ml of LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) and allowed to grow o/n at 37°C with shaking (220 rpm). 4 ml were removed and added directly to 400 ml of LB medium. The bacteria were grown to an OD_{590} of 0.375 before incubating the bacteria on ice for 10 min. The bacteria were sedimented by centrifugation at 3600xg for 7 min at 4°C. The pellet was carefully resuspended in 20 ml of ice cold 0.1 M $CaCl_2$ and allowed to stand on ice for a further 10-15 min. The cells were centrifuged again under the same conditions and resuspended in another

20 ml of ice cold $CaCl_2$. This process was repeated once more and the final pellet resuspended in 2 ml of ice cold $CaCl_2$. After a short period on ice, the bacteria were dispensed in 50 µl aliquots in pre-chilled reaction tubes and snap-frozen in liquid nitrogen before storing at -80^{0}C.

Transformation of bacteria

(a) Chemically

Depending on the application, 5 ng of supercoiled plasmid DNA or 1 µl of a ligation mix (usually a 1/10 of the ligation) was added to 50 µl of competent cells and left on ice for 30 minutes. Following this, the cells were heat-shocked at 42^{0}C for 90 seconds before chilling the tube on ice for two minutes. After addition of 1 ml of SOC medium (2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, and 20 mM glucose) the bacteria were transferred to a shaker and incubated for 45 minutes at 37°C. The cells were then pelleted by a short centrifugation (3 min at 1000xg) and 1 ml of the supernatant was removed before resuspending the cells in the remaining 200 µl. A volume of 50-200 µl was plated out on LB-agar plates supplemented with the appropriate antibiotic and the plates incubated 18-24 hours at 37°C.

(b) Electroporation

Usually, 1-2 µl of a ligation mixture was added to 40 µl of electrocompetent cells (Invitrogen BV, The Netherlands) previously aliquoted in pre-chilled reaction tubes. The cells were then immediately transferred to a pre-cooled electroporation cuvette (0.1 cm gap, BioRad) and pulsed at 1.8 kV (Gene pulser, BioRad). After the pulse, 1 ml of SOC medium was added to the cuvette and retrieved with the cells and placed in an eppendorf tube before incubation at 37°C for 60 minutes. Cells were pelleted before plating out on appropriate LB-agar plates.

Phenol/Chloroform extraction of nucleic acids

To remove protein contaminants from nucleic acids, an equal volume of Tris-buffered phenol/chloroform/isoamylalcohol (25:24:1) was added and vortexed. The two phases were separated by centrifugation at 10000xg for 15 minutes after which the upper aqueous nucleic acid containing phase was transferred to a new tube and subjected to another round of extraction with chloroform/isoamylalcohol (24:1).

Isolation/Purification of DNA from agarose gels

A number of methods were employed to isolate DNA (e.g. PCR fragments, restriction digested vectors and inserts) from agarose gels once electrophoresis was completed.

(a) Electrophoretic isolation of DNA

Once the DNA had migrated the desired distance, as determined by visualising the gel under UV light, a slit was cut into the gel with a scalpel just below the chosen DNA band. A strip of DE81 DEAE-cellulose membrane (Schleicher & Schuell, Dassel) was inserted into the slit and electrophoresis continued until the DNA fragment had run onto the membrane. At this point the membrane was removed, rinsed briefly with distilled H_2O and the DNA eluted by incubation in 400 µl 1.5 M NaCl, 10 mM Tris-HCl pH 7.5 and 1 mM EDTA for 30 min at 65°C with shaking. After two extractions with phenol/chloroform, the DNA was precipitated with ethanol, air dried and dissolved in an appropriate volume of water.

(b) EasyPure Kit (Biozyme) DNA isolation from agarose gels

As above with the exception that the gel strip containing the DNA is added to 3x it's weight to volume of salt buffer (all reagents provided in the kit). The gel piece is melted in the buffer by incubation at 55°C before the binding resin is added. After incubation for 5 min at room temperature, the binding resin is pelleted at 10000xg for 5 s, washed with washing buffer, pelleted once more and

finally after air drying taken up into 20 µl of water. To remove the resin the dissolved DNA is recovered by centrifugation at 10000xg for 5 min.

Ethanol/Isoamylalcohol precipitation of nucleic acids

The precipitation of both RNA or DNA was performed by adjusting the final salt concentration of the reaction mixture to 300 mM with 3M Na-acetate (pH 5.2) and adding 2.5 volumes of 100% ethanol followed by a 30 minute incubation at -20 °C. The precipitate was pelleted by centrifugation at 13000xg, at RT for 30 minutes after which the aqueous phase was discarded. The pellet was washed once with 70% ethanol to remove salt and allowed to air dry for 10 minutes.

Determination of nucleic acid concentration

The concentration of nucleic acids in aqueous solution can be determined by spectroscopic measurement of their extinction coefficient at 260nm and 280nm. An optical density (OD) of 1 at 260 nm is equivalent to 50 µg/ml of double stranded DNA, 40 µg/ml of RNA or 20 µg/ml single stranded oligonucleotide. The OD_{280} is used as an indication of purity and should be approximately 50% of the OD_{260} value. Pure RNA and DNA should have an OD_{260}/OD_{280} ratio of 1.8-2.0 and 1.6-1.8 respectively in bi-dest water.

Restriction digest of DNA

5 units of the enzyme per µg of DNA have been used for restriction digests. The digestion was performed using the appropriate enzyme buffer and incubation temperature as recommended by the supplier. Reactions were incubated for ≥1 hour at a final DNA concentration of 1 µg/10 µl after which the reaction was terminated by heat inactivation of the enzyme (65°C for 10 minutes) followed by phenol/chloroform extraction. The digestion was controlled by agarose gel electrophoresis.

DNA ligation

All ligation reactions were performed in a total volume of 10 µl and incubated overnight at 16°C.

(a) Ligation of PCR fragments

After purification of the PCR fragment, the DNA was directly cloned via the T/A overhangs into the T/A cloning vector (original T/A cloning kit®, Invitrogen BV, The Netherlands) according to the suppliers protocol and using the kit's components.

(b) Sub-cloning

The cloned fragment of DNA was released from the vector using appropriate restriction endonucleases, purified by agarose gel electrophoresis and subsequently cloned into the new vector using compatible sites or through blunt end ligation.

Isolation of total RNA from cells or tissue

Total RNA was prepared from cells which were 40-60% confluent in 9 cm culture dishes (Greiner, Frickenhausen). The medium was completely removed and cells were lysed by adding 550 µl of RNA solubilization buffer (4 M Guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 100 mM β-Mercaptoethanol and 0.5% sodium-lauroylsarcosine) drop by drop to cover the entire plate. The cell lysate was collected, transferred to an eppendorf tube and vortexed briefly. 60 µl of 3 M sodium-acetate pH 4.2 was added and mixed by vortexing. 550 µl of phenol (pH 4.0) was added and mixed by vortexing for 30 seconds. 150 µl of chloroform was added and again mixed by vortexing. The mixture was incubated on ice for 10 minutes and centrifuged for 15 minutes at 10000xg at 4°C. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by adding equal volume of isopropanol and kept for 30 minutes at –20°C. The samples were centrifuged at 10000xg for 10 minutes and the supernatant was discarded. The RNA pellet was dissolved in 200 µl of RNA solubilization buffer by vortexing. The RNA was reprecipitated by addition of 200 µl isopropanol and kept at –20°C for 30 minutes. The samples were

centrifuged at 10000xg at 4°C. The supernatant was discarded and the pellet was resuspended in 75% ethanol, vortexed and incubated for 15 minutes at room temperature (RT) to dissolve residual amounts of guanidinium contaminating the pellet. The samples were centrifuged for 10 minutes at 10000xg at RT. The RNA pellet was dried at RT for 5 minutes and then dissolved in 30-50 µl of freshly autoclaved water by incubating at 65°C for 5 minutes. The RNA was quantified and stored at –80°C.

RNeasy Mini protocol for RNA clean-up

Tissue total RNA was purified using the RNeasy clean-up protocol and reagents supplied (Qiagen, Hilden). 20µg of total RNA was taken and processed according to supplier's recommendations. The cleaned RNA was then eluted in a total volume of 40 µl. RNeasy clean-up was only performed on those RNA samples intended for the use of RT-PCR.

Isolation of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was prepared from cells which were 40-60% confluent in a 15 cm culture dish (Greiner, Frickenhausen). The medium was removed and the cells were washed twice with ice cold PBS. The cells were then immediately lysed in 10 ml of STE-SDS-buffer (20 mM Tris-HCL pH 7.4, 100 mM NaCl, 10 mM EDTA and 0.9% SDS) containing 300 mg/ml proteinase K (Sigma, München). The high molecular weight DNA was sheared for 10 seconds with an Ultra-Turax™ homogenizer followed by incubation at 37°C for 30 minutes. After adjusting the final concentration of NaCl to 500mM, 50-200 mg of oligo-dT cellulose (type VII, Pharmacia Biotech) swollen in STE (20 mM Tris-HCL pH 7.4, 100 mM NaCl, 10 mM EDTA and 0.5% SDS) was added and the resulting mixture rotated at least for 4 hours to allow binding of the poly(A)$^+$ RNA to the oligo-dT cellulose. The oligo-dT cellulose was collected by pouring the contents into a 20 ml Bio-Rad Econo-Pac column, allowing the liquid to drain out while retaining the oligo-dT cellulose. It was then washed with 3 x 20 ml of HSB solution (300 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA and 0.1% SDS). The poly A$^+$ RNA was eluted from the oligo-dT cellulose by addition of 5 ml of freshly autoclaved water and the contents were collected in an autoclaved corex tube and precipitated by adding 1/10 volume (500 µl) of 3 M Na-Acetate pH 5.2

and 2.5 volumes (12.5 ml) ethanol for 2 hours at -80°C, followed by centrifugation at 10000xg for 45 minutes at 4°C. The RNA pellet was washed once with 75% ethanol, and air dried for 10 minutes. The pellet was then dissolved in freshly autoclaved water. The RNA concentration was determined and stored at -80°C.

Transient transfection of 5L and BP8 cells

Cells were plated out at a density of $2.5x10^6$ per 15 cm culture dish 2 days prior to transfection. Cells were trypsinized, resuspended in DMEM supplemented with 10% FBS. In order to neutralize trypsin they were collected by centrifugation at 50xg, washed twice in DMEM (w/o FBS) prewarmed to room temperature. The cells were then resuspended in DMEM without FBS. 200 µl of cell suspension containing approximately $5x10^6$cells was mixed with DNA to be transfected. Electroporation was performed in 4 mm gap cuvettes with a pulse of 280 V and a capacitance of 500 µF in a Bio-Rad gene pulser. After electroporation, cells were allowed to recover for 10 min at room temperature and suspended in 10 ml of DMEM containing 10% FBS. For -/+ TCDD treatments (with same set of DNAs transfected) cells from two electroporations were pooled and splitted into two 9 cm culture dishes. Cells were treated with 1 nM TCDD by addition of 1µM stock solution of TCDD in DMSO or with 0.01% DMSO at indicated time points.

First and second strand cDNA synthesis

(a) First strand synthesis using SuperScript™ II for RT-PCR

For most applications, 2 µg of high quality poly $(A)^+$ RNA together with 500 ng of oligo $(dT)_{12-18}$ primer in a total volume 11 µl was heated to 70°C for 10 minutes in a thermal cycler (Perkin Elmer 9600/2400) before placing the reactions rapidly on ice. The reaction mixture was subsequently made up to 20 µl by adding 4 µl of 5x first strand reaction buffer (provided with the reverse transcriptase), 2 µl of 0.1 M DTT and 1 µl dNTP mix (10 mM each dATP, dCTP, dGTP and dTTP). Reverse transcription was started by addition of 2 µl of SuperScript™ reverse transcriptase (Gibco BRL, Eggenstein) to each reaction before incubation for 1-1.5 hours in an air incubator previously equilibrated at 42°C. The reverse transcriptase was inactivated by rapidly heating the contents

to 94°C for 3 minutes and then placing the tubes on ice. The first strand reactions were stored at -20°C once their volume had been adjusted to 200 µl by the addition of 180 µl bi-dest H_2O.
In order to monitor the efficiency of the first strand reaction, 1 µl was removed directly after the addition of the reverse transcriptase and added to 1 µl of a labelling mixture containing 0.3 µl [^{32}P]dCTP and likewise incubated at 42°C. This so called *tracer reaction* was stopped by the addition of EDTA to a f.c. of 20 mM. Specific incorporation of dCTP into high molecular weight nucleic acid was determined by the TCA precipitation procedure. Typically, ~28 % of all poly(A)$^+$ RNA transcripts were converted into first strand cDNA. Note: first strand cDNA synthesis performed for the generation of a subtracted library was done exactly as described above with one exception. The oligo (dT) primer used for cDNA synthesis contained an Rsa I restriction site and a `dT´ tail of 30 nucleotides in length. This primer was provided in the PCR-Select™ cDNA subtraction kit (Clontech Laboratories, Inc. USA).
In case of performing second strand synthesis, the heat inactivation and dilution steps were omitted and the reaction vials were placed on ice after incubation at 42°C instead.

(b) One-tube second strand synthesis

Following first strand synthesis, second strand synthesis was performed by adding 92 µl of sterile, bi-dest water, 32 µl of 5x second strand buffer [94 mM Tris-HCl, pH 6.9, 453 mM KCl, 23 mM $MgCl_2$, 750 µM ß-NAD and 50 mM $(NH_4)_2SO_4$], 3 µl of *Escherichia coli* DNA ligase (7.5 U/µl), 4 µl *E.coli* DNA polymerase I (10 U/µl) and 0.7 µl of *E.coli* RNase H (2 U/µl). The contents were mixed and 5 µl removed for the radioactive incorporation assay (as above). Both reactions were incubated at 16°C for 2.5 h. The double stranded cDNA in the unlabelled reaction was blunted by the addition of 2 µl of T4 polymerase (10 U/µl, using 10 U per µg of mRNA taken in the reaction), followed by further incubation at 16°C for 20 minutes before both reactions were stopped by addition of EDTA to a final concentration of 10 mM. As before, labelled second strand cDNA synthesis was monitored by TCA precipitation and separated on an alkaline agarose gel as described by Maniatis et al. (1989). The unlabelled fraction was phenol/chloroform extracted and precipitated in the presence of 10 µg of glycogen before resuspending the ds-cDNA in bi-dest H_2O to a concentration of 0.1-0.5 mg/ml.

Polymerase Chain Reaction (PCR)

All PCR reactions were performed in a total volume of 25-100 µl, in the presence of 250 µM dNTPs, 1-2 pmol of primer(s), 0.25U to 1U of Taq and 1x supplier's buffer and 1.5 - 2.0 mM $MgCl_2$ f.c.. The reactions were carried out in a number of commercially available PCR thermo Cyclers (e.g. Perkin Elmer 9600/2400 and DNA Engine MJ Research), using specific cycling parameters depending on the application. Analysis of the PCR reaction was performed using gel electrophoresis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

All reactions were performed as described for standard PCR with the exception that 5 µl of a previously diluted first-strand cDNA reaction was taken as template DNA and the primers used were specific for the gene of interest only (as determined through BLAST). Again, specific cycling parameters were dependent on the primers chosen and size of the expected product. Evaluation of the RT-PCR reaction was performed by gel electrophoresis. The product was sequenced after cloning when required.

Radioactive labelling of DNA / cDNA probes

Approximately 25 ng of ds cDNA in a volume of 45 µl was denatured by heating to 100°C for 5 minutes. The entire solution was then added to a *redi* Prime reaction vial (*redi* Prime kit, Amersham. Life Science) to which was added 5 µl of [α^{32}P]-dCTP (Amersham Buchler GmbH, Braunschweig, 370 MBq/ml, 10 mCi/ml). After incubation for 15 minutes at 37°C unincorporated nucleotides were removed from the labelled DNA using Sephadex G-50 columns (Pharmacia) according to the manufactures guidelines. The labelled DNA was eluted in a volume of 400 µl TE-buffer and the required amount was denatured at 100°C for 5 minutes immediately before use.

Sequencing of double-stranded template DNA

(a) [α-^{33}P]-ddNTP method

The Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham) was used for the sequencing of cloned templates. The kit combines two revolutionary innovations for sequencing DNA. First, the label is incorporated into the DNA reaction products by the use of four [α^{33}P]-ddNTP terminators and secondly, the use of an engineered Thermo Sequenase DNA polymerase which allows efficient incorporation in cycling sequencing protocols. Sequencing of desired templates was carried out according to the manufacturer's guidelines. Approximately, 0.5 µg of plasmid DNA was taken together with 1-2.5 pmol of an appropriate primer (usually M13 Forward or Reverse universal primers) with 8 U of the Thermo Sequenase polymerase in a total volume of 20 µl. From this mixture 4.5 µl was aliquoted into each termination vial (G, A, T and C) and cycled in a Perkin Elmer 9600 PCR machine using the following cycle parameters: 95°C, 30 s, 55°C, 30 s and 72°C, 1 minute for a total of 35 cycles. Upon completion, 4 µl stop solution was added and 3 µl of the mix was loaded per lane and resolved over a 6% polyacrylamide 6 M urea TBE gel. Once the run had reached the desired length, the gel was removed, dried on Whatman 3MM paper at 80°C for 2 hours on a vacuum gel dryer before autoradiography. Films were developed after 18-36 hours exposure.

(b) ™Vistra DNA sequencer 725

For the use of automated fluorescent sequencing of template DNA, a commercially available kit was used (Thermo Sequenase pre-mixed cycle sequencing kit, Amersham) together with the appropriate Texas Red labelled primers. All reagents were taken from and used following the supplier's protocol. The thermocycling conditions for the sequencing reactions were set at: 94°C 1 minute, then 22 cycles of 94°C, 30 s; 50°C, 15 s; and 72°C for 30 s. Following completion, 3 µl of loading dye was added to each reaction vial and mixed before placing the reactions in a vacuum centrifuge until the desired volume had been reached (usually 3-4 µl). After the gel had been pre-run for 30 minutes 3 µl of each reaction sample was loaded onto the gel and allowed to run overnight. Sequences were analysed using the provided software.

Generation of a subtracted library by SSH

Suppression Subtractive Hybridization was performed between non treated 5L cells (driver) and 4 h TCDD treated 5L cells (tester), using the PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories, Inc. USA) according to the manufacturers' recommendations. All PCR and hybridization steps were performed on a Perkin-Elmer 9600 thermal cycler. Double-stranded cDNA was synthesized from 2 μg of poly(A)$^+$ RNA of the non treated or 4 h TCDD treated cells as described earlier. Both ds cDNAs were then Rsa I digested, precipitated and redissolved in freshly autoclaved water according to Clontech cDNA subtraction user manual. Tester cDNA was ligated with adaptors provided in the kit. The ligation efficiency was verified by comparing PCR products obtained with gene specific primers for GAPDH or one GAPDH specific primer and another adaptor specific primer. The difference between the intensities of the above PCR reactions was less than two fold. All manipulations were performed in accordance with the protocol in the user manual.
For the first hybridization the mixture of driver and tester cDNAs was denatured at 98°C for 90 seconds and then incubated at 68°C for 11 h. For the second hybridization, fresh driver cDNA was denatured at 98°C for 90 seconds then the samples from the first hybridization were mixed together without denaturing in the presence of freshly denatured driver cDNA and incubated at 68°C for 20 hours. At the end of the incubation period 200 μl of dilution buffer (20 mM Hepes-HCl (pH 8.3), 50 mM NaCl and 0.2 mM EDTA (pH 8.0)) was added. Differentially expressed cDNAs were selectively amplified in two rounds of PCR as described in the user manual provided in the subtraction kit.

Evaluation of subtraction efficiency

Equal amounts of amplified cDNA from the tester and the subtracted library cDNA were resolved on a 2.0% TAE agarose gel, blotted and transferred onto nylon membrane as described later under southern blotting. This was performed in order to monitor the degree of subtraction efficiency as determined by the removal of genes common to both populations (e.g. GAPDH) and the subsequent enrichment of genes specific to the tester population (e.g. CYP450 1A1 and GST-Ya). Hybridizations were carried out as mentioned in southern blotting and hybridization analysis. After washing the membranes were sealed in a plastic bag and exposed to Amersham hyper film MP at -80°C.

Cloning into TA vector

After evaluation of the subtraction efficiency the subtracted PCR products were cloned into pCR2.1 vector (TA cloning kit, Invitrogen BV, The Netherlands). The subtracted PCR products were incubated for 30 min at 72°C with additional dATP and Taq DNA polymerase (Eurobiotaq) to ensure that most of the cDNA fragments contained 'A-overhangs' as the initial PCR amplification of subtracted products was done with Klen Taq polymerase mix (Clontech Laboratories, Inc. USA) which contained proof-reading polymerases. The subtracted PCR products were precipitated and approximately 100 ng of the cDNA was ligated into 50 ng of linearized vector having 3' T-overhangs provided in the kit. One third of the ligation products were introduced into electro-competent bacteria by electroporation in 2 mm gap cuvette using an E.coli pulser (Bio-Rad) at 1.8 kV. The library was plated onto 15 cm agar plates containing 100 µg/ml ampicillin, 100 µM IPTG and 50 µg/ml X-Gal. Plates were incubated at 37°C until colonies were visible and then incubated further at 4°C until blue/white staining could be clearly distinguished.

4.3 Analytical methods

Northern blot hybridisation

10 to 20 µg of total RNA or 2-5 µg poly $(A)^+$ RNA was diluted with freshly autoclaved bi-dest H_2O to give a final volume of 11.25 µl. To this was added 38.75 µl of formaldehyde denaturing buffer (25 µl formamide, 5 µl 10xMOPS (200mM MOPS, 50mM EDTA, pH 7.0) running buffer and 8.75 µl of 37% formaldehyde) and the samples were mixed well by vortexing. The samples were incubated at 55°C for 15 minutes before addition of 10 µl of RNA loading buffer (1mM EDTA, 50% glycerol, 0,1% bromphenolblue). The RNA samples were then size fractionated on a 1% formaldehyde/MOPS agarose gel. 1% previously autoclaved agarose in 1xMOPS buffer was melted in a microwave oven and allowed to cool to 60°C before addition of formaldehyde to a f.c. of 3.5% and 1 µl of ethidium bromide (10 mg/ml). Electrophoresis was done at 100 V at RT for 2 h in 1xMOPS running buffer. The integrity of the RNA was visualised under 260 nm UV light source. The RNA was then transferred to Hybond-N+-membrane (Amersham Buchler GmbH, Braunschweig) using 20xSSC (3 M NaCl, 0.3 M Na_3citratex$2H_2O$) as the transfer buffer. After the o/n

transfer the membrane was rinsed with 5xSSC and the RNA was covalently cross-linked to the active OH groups in the membrane with UV (UV stratalinker 2400, Stratagene, La Jolla, CA, USA).
The hybridization procedure was carried out as follows: the membrane was prehybridized in a glass tube for 15 min at 65° C in 4xSSC, 0.02% Ficoll 400, 0.02% polyvinylpyrollidone, 0.02% BSA, 3% Pippi (0.5 M sodiumphosphate pH 7,3, 1.5% sodiumpyrophosphate) and 0.1% SDS in a hybridization oven (Amersham Buchler GmbH, Braunschweig). Salmon sperm DNA was added then to block the unspecific hybridization to a final concentration of 20 µg/ml and prehybridization was continued for another 45 minutes. Hybridization was carried out in 4xSSC, 10mM EDTA and 0.1% SDS containing $1x10^6$ cpm/ml of random primer labelled DNA probe (to which 20 µg/ml of salmon sperm DNA was added and denatured at 95°C for 10 minutes) for about 12 h at 65°C. The membrane was washed three times at 65°C in 2xSSC (1 h), 1xSSC (30 min) and 0.5xSSC (15 min) respectively each containing 0.1% SDS and 3% Pippi.
The membrane was sealed in a plastic bag, exposed to a phosphoimager plate for quantification (PhospholmagerFujix BAS 2000) and then later exposed to Amersham hyper film MP at -80°C with an intensifying screen.

Immobilization of cDNAs on a Hybond-N⁺-membrane via slot blot

A Hybond-N+-membrane was rinsed in water and fixed together with a piece of 3MM-paper soaked in 10xSSC in a slot blot chamber (Schleicher&Schuell, Dassel). 50-100 ng of DNA in 30 µl were mixed with 20 µl of loading buffer (0.3M NaOH, 15% Ficoll 400, 0.1% bromphenolblue). DNA mixture was soaked onto the membrane, washed two times with 300 µl 0.3 M NaOH and rinsed in 5xSSC. Membrane was air dried and DNA was cross-linked with UV (UV stratalinker 2400, Stratagene, La Jolla, CA, USA). Prehybridisation and hybridisation was done like given in the southern blotting procedure below.

Southern blotting and hybridization analysis of PCR products

The PCR products were loaded onto an agarose gel and run in 1xTAE buffer until the DNA had migrated sufficiently as determined by visualisation under UV-light. The gel was then incubated in a solution of 0,4 M NaOH for 20 min and shaken slowly on a platform shaker. The DNA was transferred to Hybond-N+ membrane as described in Current Protocols in Molecular Biology using 0.4 M NaOH as the transfer buffer. After the overnight transfer, the membrane was rinsed in 5xSSC and stored dry if not used immediately. The membrane was prehybridized in a glass tube for 1 h at 65°C in church buffer (1.0 M $NaPO_4$, pH 7.2, 7% SDS) (Church and Gilbert, 1984)). The hybridization was also carried out in church buffer containing $1x10^6$ cpm/ml of random primer labelled DNA probe (to which 20 µg/ml of salmon sperm DNA was added and denatured at 95°C for 10 minutes) for about 12 h at 65°C. The membranes were washed three times for 30 min each at 65°C in 2xSSC, 0.2xSSC and 0.1xSSC containing 0.1% SDS each. The membranes were sealed in a plastic bag and exposed to Amersham hyper film MP at -80°C.

Immunoblot analysis of proteins (Western blot)

(a) Separation of proteins by polyacrylamide gel electrophoresis (PAGE; Laemmli, 1970)

The polyacrylamide gel consisted of a 12% separating gel and a 5% stacking gel. Reagents for the separating gel were 2 ml of acrylamide/*bis*-acrylamide (30:0.8, Carl Roth GmbH & Co, Karlsruhe), 1.3 ml of 1.5 M Tris-HCl pH 8.8, 25 µl of 20% SDS, and 1.7 ml of H_2O. To the mixture 50 µl of 10% ammonium persulphate (APS) was added and the reaction initiated with 5 µl of TEMED. The gel mix was poured between two glass plates with spacers in between (Mighty small II, Hoefer) and allowed to polymerize for 30 min. Then, a stacking gel was poured on top. This was made up of 330 µl of acrylamide/*bis* - acrylamide, 250 µl of 1M Tris-HCl pH 6.8, 10 µl of 20% SDS, 1.4 ml of H_2O, 200 µl of 10% APS and 2 µl of TEMED. The gel was run in 1x Laemmli-running buffer (25 mM Tris-HCl pH 8.3, 0.2 M glycine and 0.1% SDS) until the desired distance had been reached.

(b) Preparation of cell extracts

Cells were grown logarithmically on a 9 cm culture dish. Before harvesting cells were washed once in PBS, lysed in 2xLaemmli sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 2% ß-mercaptoethanol, 0,01% bromphenolblue) and sonified with a Branson sonifier.
Before loading the samples, the extracts were denatured by heating for 5 minutes at 95°C, chilled on ice and loaded onto the gel. The electrophoresis was carried out at 100 V for 2 hrs.

(c) Determination of protein contents by Coomassie blue staining

The part of the gel that was not used for transfer was cut and incubated with mild shaking for 1-2 hours in Coomassie staining solution (0.25% Serva Blue G250, 46% methanol, and 8% acetic acid). Afterwards the gel was destained in two steps. First in 50% methanol and 10% acetic acid and then in 5% methanol with 7% acetic acid until blue clear bands were visible. The gel was then removed and vacuum dried.

(d) Electrophoretic transfer of resolved proteins onto membrane and subsequent antibody detection

The section of the gel containing proteins of interest was isolated and a piece of Immobilon membrane (Millipore, type PVDF, pre-soaked in methanol, water, transfer buffer (200 mM glycine, 25 mM Tris-base, 20% methanol)) placed on top of the gel. The proteins were transferred in transfer buffer using a BioRad Trans-Blot Cell transfer chamber. Transfer was usually carried out at 65 mA for one hour using semidry blot. The membrane was then incubated for 30 minutes with blocking buffer (5% low-fat milk in TBS (20 mM Tris-base, 150 mM NaCl, pH 7.6) with 0.2% Tween 20 to reduce unspecific binding of the antibodies to the membrane. For detection of proteins of interest the membrane was further incubated in TBS with 0.2% Tween 20 buffer containing the appropriate primary antibody (at a dilution of 1:1000) for one hour at RT or at 4°C for about 12 hours. After extensive washing in TBS/0.2% Tween 20, the membrane was incubated again in TBS with 0.5% Tween 20 containing a 1:2000 dilution of a peroxidase-coupled anti-rabbit secondary antibody. Once the membrane had been washed 3 times 5 min each in TBS/0.2% Tween 20, detection of specific proteins was

achieved by enhanced chemifluoresence using *Amersham ECL Western blotting detection reagents* (RPN 2106) following the manufacturer's instructions.

(e) antibodies used

Anti p27-KIP (rabbit polyclonal antibody, sc 528, Santa Cruz, Heidelberg)

Anti ERK1 (rabbit polyclonal antibody, sc 094, Santa Cruz, Heidelberg)

Anti-rabbit immunoglobulins (polyclonal IgGs, peroxidase-coupled, from goat, DAKO, Glostrup, Denmark)

DNA-staining procedure for hepatocytes

Logarithmically growing cells were washed once with PBS ($-Ca^{2+}/Mg^{2+}$), trypsinized and collected in DMEM medium containing 10% FBS. The cells were collected by centrifugation at 250xg.
After washing once in PBS, the cells were resuspended in 2ml PBS and an equal volume of fixation solution (8% formaldehyde, 2% methanol dissolved in PBS) was added. Cells were incubated for 10 min at RT and permeabilized by addition of 400 µl 0.5% Tween 20 for another 10 min. 10 ml of ice cold PBS/5% FBS was added and cells were collected at 800xg. For DNA-staining cells were resuspended in PBS/5% FBS containing the DNA bisbenzimide dye H33258 (1 µg/ ml, Hoechst).

Immunofluorescence assay for the detection of 5-Bromo-2´-deoxy-uridine (BrdU) incorporated into cellular DNA (kit by Boehringer Mannheim)

32h after transfection cells were treated with 1nM of TCDD or 0.1% DMSO for additional 18h of which during the last 5h 10 µM BrdU was present in the culture medium. Cells were harvested using trypsin and green fluorescing cells were sorted to a purity greater than 80% by FACS (Becton Dickinson, FACS Star Plus). Sorted cells were spun onto glass slides for immunocytochemical staining. Slides were washed three times in washing buffer and fixed in 70% ethanol (in

50 mM glycine buffer, pH 2.0) for at least 20 min at -20°C. After three times washing cells were covered with anti-BrdU-working solution and incubated for 30 min at 37°C. Again cells were washed three times and incubated with anti-mouse-Ig-fluorescein working solution and incubated at 37°C for 30 min. Finally cells were washed three times, covered with a mounting medium and examined in a fluorescence microscope.

Cell cycle analysis by flow cytometry

Cells were analysed by using a flow cytometry assisted cell sorter (FACS Star Plus, Becton Dickinson) having an argon laser for measuring the DNA dye H33258 (Hoechst) at 350-360 nm (extinction) and 425±22 nm (emission), GFP at 488 nm (extinction) and 530±15 nm (emission) and red autofluorescence of the cells at 488 nm (extinction) and 575±13 nm (emission). Data analysis was done using the supplied software (Cell Quest).

Measurements of Firefly luciferase activity

The medium was removed and cells were washed twice in PBS without Ca^{2+} and Mg^{2+}. The PBS was removed from cultures and 200 µl of lysis buffer (0.1M Tris acetate , pH 7.5, 2mM EDTA, 1% Triton-X-100) was added. The cultures were kept on ice for 10 min and occasionally rocked to distribute the buffer evenly over the plates.
The cell lysates were collected with a policeman into precooled eppendorf tubes and the cleared supernatant was collected after 5 min of centrifugation at 12000xg. Luciferase activity was measured by a luminator (Berthold, Wildbad). The lysate was transferred into a reading tube with autoinjection of 350 µl of assay buffer (1mM DTT, 1mM ATP in glycylglycine buffer (25mM GlyGly, 15mM $MgSO_4$ and 4mM EGTA) and 100 µl of luciferin assay solution (1mM luciferin stock solution (0.28 mg/ml) in glycylglycine buffer diluted 1:5).

Measurement of Renilla luciferase activity together with Firefly luciferase activity out of the same cell lysate (J.C. Matthews et al., 1977)

Transfected cells were washed with PBS without Ca^{2+} and Mg^{2+} and lysed in 200 µl passive lysis buffer (Promega, Mannheim). 100 µl of lysate were analyzed for firefly luciferase activity (see above). To determine renilla luciferase activity 10 µl of lysate were mixed with 390 µl of reaction buffer (0.1 M KPi-buffer (0,2 M KH_2PO_4 and 0,2 M K_2HPO_4 , pH 7,6), 0.5 M NaCl, 1 mM EDTA) together with 100 µl of 5x substrate solution (125 nM coelenterazine (Biosynth AG, Gstaad, Schweiz) in reaction buffer). After vortexing luminescence was measured by a luminator (Berthold, Wildbad).

Oligonucleotides

All oligos were obtained from either MWG Biotech GmbH or from Birsner & Grob-Biotech GmbH and were all of a HPLC purified grade.

For Sequencing

(i).	5`- GTTTTCCCAGTCACGAC-3´	PUC/M13 For-Texas red
(ii).	5`- CAGGAAACAGCTATGAC-3´	PUC/M13 Rev-Texas red
(iii).	5`- TAATACGACTCACTATAGGG-3´	T7-Texas red
(iv).	5`- ATTAACCCTCACTAAAG-3´	T3-Texas red

For PCR

(i).	5`- ACCACAGTCCATGCCATCAC-3´	GP5
(ii).	5`-TCCACCACCCTGTTGCTGTA-3´	GP3
(iii).	5`-GCCCAAGTCTGTGAAACTACTTGATTTCC-3´	106.1
(iv).	5`-GACCCAGCGGTGGAGTAGTGCTGTC-3´	106.2
(v)	5`-TGGCTCCTGGCTGCAGCAGGAGTGG-3´	GST-YA 5
(vi)	5`-GCAGAGGGAAAGAGGTCAGAAGGCTGGC-3´	GST-YA 3
(vii)	5`-ATGCATCCCTTCTACACTCGG-3´	BTG1.1
(viii)	5`-CTATATCTTAACCTGATACAGTC-3´	BTG1.2
(ix)	5`-AGCCCGAGCCTGGAGCGGATGGACGCC-3´	Kip1.5
(x)	5`-GGCCAGGCTTCTTGGGCGTCTGCTCCACAG-3´	Kip1.3

Northern probes

(i) **GAPDH**: 1.3 kB Pst I fragment from the plasmid pGAPDH-13 (Fort et al., 1985)

(ii) **CYP1A1**: Pst I -digestion of pSV4501A1 (Dogra et al., 1990) generated an approximately 400bp fragment which was gel purified and used as a probe

(iii) **GSTya**: GSTya cDNA probe was generated by RT-PCR from 5L cell RNA using primers GST-YA 5 and GST-YA 3

(iv) **p27**: rat Kip1 cDNA probe was generated by RT-PCR from 5L cell RNA using primers corresponding to nt 28-54 and nt 548-577 of the murine cDNA

Plasmids

pCMV5: (Weiß et al., 1996)

pCMV5-hGFP: Xho I/Hind III open reading frame from GFP (Heim et al.,1995) was subcloned into Sal I/Hind III digested pCMV5.

pCMV5-AhR1-805: pmu-AhR (passed by M. Göttlicher) was Sal I digested and partially filled in by Klenow polymerase, digested with Nhe I to release AhR1-805 cDNA. This was inserted into pCMV5, which was Bgl II digested, partially filled in by Klenow polymerase and again digested by Xba I.

pCMV5-Ahr1-605: pmu-AhR was digested with Pst I, 3´-overhangs were removed by Klenow enzyme and digested again which BamH I to release AhR cDNA 418-605.
This fragment is ligated into Sma I and BamH I digested CMV5-AhR1-805 (which releases cDNA portion of AhR 418-805) to get AhR 1-605.

pCMV5-AhR1-408: pmu-AhR was digested with Sal I, filled in by Klenow polymerase and digested with Bgl II to generate AhR1-408 fragment. This was ligated into pHook 2 (invitrogen, Netherlands), which was digested with Hind III, filled in by Klenow polymerase and then digested by Bgl II. The cDNA fragment of AHR1-408 containing the stop codon was released from pHook 2-AhR-1-408 by Hind III/BamH I digestion and ligated into Hind III/BamH I digested pCMV5.

pCMV5-AhRm39: passed by M. Göttlicher

pCMV5-ARNT: Cla I/Xba I released fragment from plasmid pCMV4-ARNT (Weiß et al., 1996) was subcloned into Cla I/ Xba I digested pCMV5.

pCMV5-ΔbHLH-ARNT: pBS-ΔbHLH-ARNT (Weiß et al., 1996) was BamH I/Sal I digested and subcloned into Bgl II/Sal I digested pCMV5.

pCMV5-antiKip1: the antisense expression vector was generated by cloning the PCR-product used as a northern probe into pCMV5.

5. Literature

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