AN ABSTRACT OF THE THESIS OF

Byung-Woo Ryu for the degree of Doctor of Philosophy in Toxicology presented on December 13, 1996. Title: Characterization of Pyruvate Carboxylase Responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin Exposure in C57BL/6J Male $Ah^{d/d}$ Mice.

Abstract approved: Redacted for Privacy

Henry W. Schaup

The halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental contaminant, deregulates gluconeogenesis by suppression of pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK). Mouse hepatic pyruvate carboxylase (PC), one of the rate controlling enzymes of gluconeogenesis is down-regulated in mice exposed to TCDD. It is generally accepted that critical TCDD responses are mediated by an intracellular protein called that aryl hydrocarbon receptor (AhR). In this thesis I have investigated the molecular mechanism of action of TCDD with respect to AhR involvement in the regulation of mouse hepatic PC. Here I have undertaken investigations designed to develop a better understanding of TCDD effects on expression of PC at the level of transcription. Here I report evidence for the involvement of AhR in mediation of PC reduction in response to TCDD exposure using a congenic C57BL/6J $Ah^{d/d}$ mouse strain. These mice have an AhR with a ~10 fold lower affinity for TCDD when compared to $Ah^{b/b}$ mice. Here I also report the characterization of the PC gene regulatory zones that responded to TCDD treatment. This was accomplished using a transient

expression assay in tissue culture (wild-type mouse hepa1c1c7 cell) studies.

If the PC/TCDD response is AhR mediated, congenic $Ah^{d/d}$ mice would require much higher dose of TCDD to suppress PC. In $Ah^{d/d}$ mice, a 60-fold higher dose of TCDD was necessary to produce a PC/TCDD effect. The different response in PC mRNA reduction caused by TCDD exposure between $Ah^{b/b}$ and $Ah^{d/d}$ mice was observed and provides evidence for AhR involvement in that repression. To gain insight into the molecular mechanism by which TCDD modulates effects on PC gene transcription, I cloned the $Ah^{d/d}$ mouse PC gene regulatory zones (upstream promoter and intron 1 of the PC gene) using an inverse polymerase chain reaction (IPCR). The functional roles of the cloned PC gene regulatory zone and their involvement in responses to TCDD exposure were analyzed using a transient transfection assay. In this thesis, I report the DNA sequences of the cloned regions of the PC gene regulatory zones and the characterization of the PC regulatory zone responses to TCDD treatment. The PC gene has a TATA-less promoter which is fully functional for initiation of transcription by RNA polymerase II. In contrast to animal studies, the transient expression assay, using a luciferase gene driven by the PC promoter in Hepa1c1c cells, produced an induction of the PC gene in the presence of TCDD. A polypyrimidine/polypurine sequence binding DREs found in intron 1 of the PC gene was not conclusively shown to be involved in regulation of the PC mRNA transcription.

Characterization of Pyruvate Carboxylase Responses to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Exposure in C57BL/6J Male $Ah^{d/d}$ Mice

by

Byung-Woo Ryu

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Doctor of Philosophy

Completed December 13, 1996
Commencement June, 1997

Doctor of Philosophy thesis of Byung-Woo Ryu presented on December 13, 1996

APPROVED:

Redacted for Privacy

Major Professor, Representing Toxicology

Redacted for Privacy

Chair of Program of Toxicology

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Byung-Woo Ryu, Author

Acknowledgments

The work presented in this thesis would not have been possible without the love and support of a great many people. First and foremost is my family, especially my parents, Young-Sang Ryu and Song-Soo Shin.

I am very grateful to my major professor, Dr. Henry W. Schaup, for his intensive help and guidance throughout the research and preparation of the thesis. I would like to express my appreciation to my graduate committee: Dr. Donald R. Buhler, Dr. Ian J. Tinsley, Dr. Nancy Kerkvliet , and Dr. Linda L. Blythe for their time and suggestion. I also would like to acknowledge Dr. Kevin Ahern for his great help and suggestion for my research.

During graduate school, I have been blessed with the friendship of very special people; Sirinmas Intharapanith, Dr. Barney Sparrow, Cynthia Thompson, and Shukla Roy.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

Characterization of Pyruvate Carboxylase Responses to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Exposure in C57BL/6J Male $Ah^{d/d}$ Mice.

Introduction

1.1 Introduction

1.1.1 Toxicity of TCDD

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin(TCDD) is the most potent toxic chemical among 75 possible chlorine-substituted dibenzo-*p*-dioxin isomers (Schwetz *et al.* 1973). Physical and chemical properties for TCDD are melting point; 305-306°C, water solubility; 1.93E-05 mg/l, vapor pressure; 7.40E-10 mmHg, Henry's constant; 1.6E-05 atm-m^3/mol, and Log K_{ow}; 6.64. TCDD is a by-product of industrial processes involving chlorine chemistry and the combustion of chlorine containing fuels in most products. The toxic effects of TCDD vary with the dose, route of exposure, length of exposure, and animal species (Poland and Knutson 1982). Variable TCDD toxicity in different animal species is well documented and exemplified by the fact that the acute oral LD_{50} of TCDD varies over 5000-fold range in different species. For example the LD_{50} for guinea pig is 1 µg/kg (Schwetz *et al.* 1973) and for hamster it is 5000 µg/kg (Olson *et al.* 1980; Henck *et al.* 1981). To some extent, the toxicity of TCDD is dependent on body burden. Because of its chemical and physical properties (low vapor pressure and water solubility, high lipophilicity and relative stability), TCDD tends to persist and bioaccumulate. It has relatively long half-life in animal and human tissues (Mackay *et al.* 1992). TCDD has a half-life of approximately 10-15 days in mouse and 5-10 years in humans (Birnbaum 1986; Pirkle *et*

al. 1989). Due to the long half-life, the low dose of chronic exposures as well as acute to TCDD provide sufficient body burden resulting in the typical toxic endpoints (DeVito and Birnbaum 1994).

Although there are species differences in the toxic effects of TCDD, several responses are commonly manifested. Examples of these are wasting syndrome (Max and Silbergeld 1987; Peterson *et al.* 1984), thymic atrophy (Vos *et al.* 1991), chloracne (McConnel and Moore 1979), hepatotoxicity (Mocarelli *et al* 1991), immunotoxicity (Kerkvliet 1994), reproductive toxicity (Kociba *et al.* 1976; Umbreit *et al.* 1987), developmental toxicity and teratogenicity (Birnbaum 1991), and carcinogenicity (Fingerhut *et al.* 1991). Upon exposure to LD_{50}, animals begin to die within a few weeks. The exact molecular mechanism inducing death is unclear but severe weight loss always precedes death. Rapid reduction of muscle mass and adipose tissue is manifest in wasting syndrome (Max and Silbergeld 1987; Peterson *et al.* 1984), There is growing evidence indicating that TCDD might cause reduced food intake in animals by affecting the central nervous system, in particular, the hypothalamus. The hypothalamus regulates balance in food intake (Peterson *et al.* 1984).

TCDD treatment at sub-lethal doses cause thymic atrophy in all experimental animals studied (Vos *et al.* 1991). The thymus has no significant function in adult animals but is critical in the development of the immune system. Therefore, thymic atrophy caused by TCDD in adult animal doesn't change immune function. However, the thymus is an extremely sensitive target organ in developmental stage of animal (Vos and Moore 1974).

Chloracne is acne induced by chlorinated organic compounds. This disease is uniquely induced by polyhalogenated aromatic hydrocarbon.

Both dermal and systemic exposure to TCDD can cause chloracne in humans, monkeys, hairless mice, and on rabbit ears (McConnell and Moore 1979),

Although there is variation in the degree of toxicity among animal species, hepatotoxicity is a commonly observed and this may contribute to TCDD lethality. Hyperplasia and hypertrophy of parenchymal cells caused by TCDD exposure produces hepatomegaly in animals treated with dioxin. Hepatic damage markers serum glutamic-oxaloacetic transaminase(SGOT) and serum glutamic-pyruvic transaminase(SGPT) activities are increased upon treatment with TCDD (Zinkl *et al.* 1973).

TCDD displays significant immunotoxic activity in animals. Humoral and cell mediated immune suppression is seen with TCDD at low doses (Vos *et al.* 1973). However, It is difficult to fully characterize TCDD-induced immunotoxic syndrome because immune responses to TCDD exposure depend on animal species, dose of TCDD, and the exposure paradigm studied (Kerkvliet 1994).

Reduced fertility, litter size, and uterine weights are observed in mice, rats, and primates exposed to TCDD (Kociba *et al.* 1976; Umbreit *et al.* 1987), An antiestrogenic action of 2,3,7,8-TCDD is responsible for the reproductive toxicity in female animals (Barsotti *et al.* 1979). Decreased serum androgen concentration is associated with male reproductive toxicity (Moore *et al.* 1985). Decreased spermatogenesis, abnormal testicular morphology, reduced testis size and accessory sex organ weight are distinctively observed symptom in TCDD exposed male animals (Chahoud *et al.* 1989).

Dioxins are also associated with developmental toxicity and teratogenicity in rodents (Birnbaum 1991). The processes of fetus

development are delicately regulated by the interaction of various hormones and growth factors. Exposure to steroid "hormonelike" chemicals such as TCDD disturbs those processes and produces deleterious effects (Couture *et al.* 1990).

Exposure to TCDD and its congeners is associated with an increased cancer risk (Fingerhut *et al.* 1991). It is not likely that TCDD is a direct mutagenic compound because DNA adducts of TCDD are not detected by very sensitive methods (Wassom *et al.* 1977; Turteltaub *et al.* 1990). Instead, TCDD is strong promoter rather than initiator in carcinogenicity. TCDD is one of the few non-genotoxic carcinogens (Pitot *et al.* 1980).

1.1.2 TCDD Disruption of Intermediately Metabolism

There have been numerous reports that TCDD causes impairment of many metabolic pathways in mice and rats (Jones *et al.* 1987; Weber *et al.* 1987; Lentnek *et al.* 1991). Of particular interest is the activity and protein level of phosphoenolpyruvate carboxykinase (PEPCK). This enzyme is one of the glucocorticoid dependent enzymes involved in the gluconeogenic pathway. TCDD treatment in rats significantly lowered levels of this enzyme in the liver (Weber *et al.* 1992; Stahl *et al.* 1993). Pyruvate carboxylase (PC) is another key enzyme that plays a pivotal role in the citric acid cycle and gluconeogenesis. PC catalyzes the formation of oxaloacetate (Figure 1.1). PC deficiency in humans result in death in utero or shortly after birth. Death is caused by lactate acidosis (Robinson *et al.* 1984; Rutledge *et al.* 1989). PC catalyzes pyruvate to oxaloacetate which is equally distributed into citric acid cycle in mitochondria and gluconeogenesis in cytoplasm (Martin *et al.* 1993). For the gluconeogenesis, oxaloacetate carbon units are converted to malate by mitochondrial malate dehydrogenase (MDH).

Figure 1.1 Schematic drawing of proposed TCDD induced enzymatic disruption of the citric acid cycle and gluconeogenesis. TCDD blocks the reactions catalyzed by pyruvate carboxylase causing reduced entrance of carbon units via oxaloacetate into the citric acid cycle clearly diminishing production ATP. Reduced mitochondria oxaloacetate levels in turn produces a shortage cytoplasmic oxaloacetate crosses the mitochondrial membrane through via the shunted malate. In the cytoplasm, TCDD also blocks the reaction catalyzed by phosphoenolpyruvate carboxykinase, thus blocking gluconeogenesis.

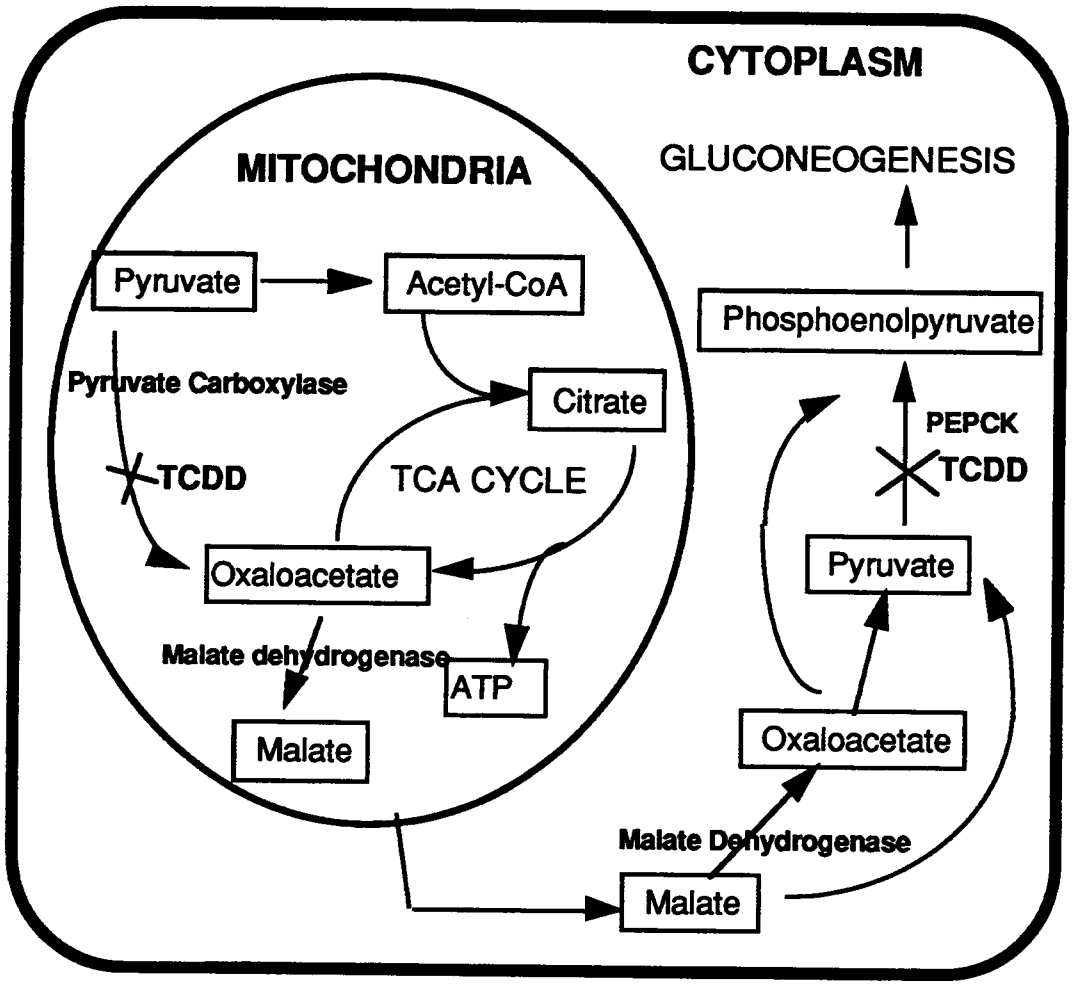

CYTOPLASM
MITOCHONDRIA
GLUCONEOGENESIS
Pyruvate
Acetyl-CoA
Phosphoenolpyruvate
Pyruvate Carboxylase
Citrate
PEPCK
TCDD
TCDD
TCA CYCLE
Pyruvate
Oxaloacetate
Malate dehydrogenase
ATP
Malate
Oxaloacetate
Malate Dehydrogenase
Malate

Figure 1.1

This malate is able to cross the mitochondrial membrane (malate shunt) to cytoplasm where cytosolic malate dehydrogenase changes it back to oxaloacetate. In the cytoplasm, oxaloacetate is a substrate for the PEPCK in the rate limiting step in gluconeogenesis. By exergonic decarboxylation of oxaloacetate, PEPCK produces phosphoenolpyruvate, a high energy intermediate compound which is a central intermediate in gluconeogenesis and glycolysis.

TCDD exposure at the dose of 20 μg/kg body weight in mice suppresses PC activity and enzyme level resulting in the accumulation of pyruvate, alanine, and lactate (Sparrow *et al.* 1994). The reduced entry level of oxaloacetate into citric acid cycle and gluconeogenesis contributes to the development of lactate acidosis (Figure 1.1). The suppression of PEPCK and PC as well as induction of lactate acidosis by TCDD exposure may also contribute to the metabolic toxicity in mice.

1.1.3 Molecular Mechanism of TCDD Signal Transcription Pathway

The aryl hydrocarbon receptor (AhR) is an intracellular protein known as a transducer that is a DNA binding transcription factor in the TCDD signal transduction pathway. AhR has a mechanism of action similar to the steroid hormone receptor with regard to ligand binding, signal transduction, and DNA binding (Evans 1988). It is generally considered that most, if not all, of TCDD toxic effects are mediated by AhR (Scheuplein *et al* 1991). Cloning and sequencing of the Ah receptor gene revealed that the Ah receptor is a member of basic helix-loop-helix containing proteins (Burbach *et al.* 1992). This basic helix-loop-helix (bHLH) region is involved in protein-protein interaction allowing the AhR to associate with Ah receptor nuclear transferase (ARNT) forming a heterodimer (Reyes *et al.* 1992). Arnt is also a

bHLH protein. The heterodimer form is able to bind *cis*-elements located in upstream zone of TCDD responsive genes. When the AhR and ARNT heterodimer bind to the dioxin response element (DRE), DNA changes its conformational structure increasing other transcriptional factor accessibility to bind the DNA. These events alters the transcription frequency of TCDD responsive genes. Alteration in gene transcription is presumed to underlie the toxicity and carcinogenicity of TCDD.

One of the best characterized responses to TCDD is the up-regulation of CYP1A1 protein (Figure 1.2) (Whitlock 1990). CYP1A1 is one of a mono-oxygenase enzyme family which detoxifies or activates endogenous xenobiotics. Upon binding to Ah receptor, TCDD activates the Ah receptor causing dissociation of two other proteins, heat shock protein 90 and a protein whose molecular mass is 50 kD designated p50 (Perdew 1992). Dissociation of those proteins accelerates TCDD liganded Ah receptor formation of an AhR:ARNT heterodimer. This is the activated form of the receptor which binds to a specific site of DNA, the DRE or xenobiotic responsive element (XRE) (Hoffman *et al.* 1991; Denison *et al.* 1988). Upon binding of the Ah receptor complex to these responsive elements, there is an increase in transcription of *Cyp1A1* gene (Whitlock 1990).

1.1.4 Ah receptor mediation of TCDD toxicity

There are two lines of evidence demonstrating the Ah receptor mediation of TCDD toxicity. Structure-activity relationship among dioxin congeners shows a direct relationship between binding affinity to the Ah receptor and the potency of the chemical to induce toxicities. The greater affinity of chemical for the Ah receptor shows the more potent toxicity (Safe 1990). The other approach has been involved mice that have different

Figure 1.2. Mechanism of action of TCDD in CYP1A1 induction. TCDD crosses the cellular membrane and binds to the Ah receptor. Heat shock proteins (HSP90) and p50 are dissociated from the Ah receptor upon binding TCDD. The TCDD liganded Ah receptor then associates with ARNT protein. The complex then interacts with the sequence specific *cis*-element of DNA designated dioxin responsive elements (DRE). When the Ah receptor binds to DNA, the transcription rate of the genes downstream of the responsive element is enhanced. The increase in transcription results ultimately increases the concentration of CYP1A1.

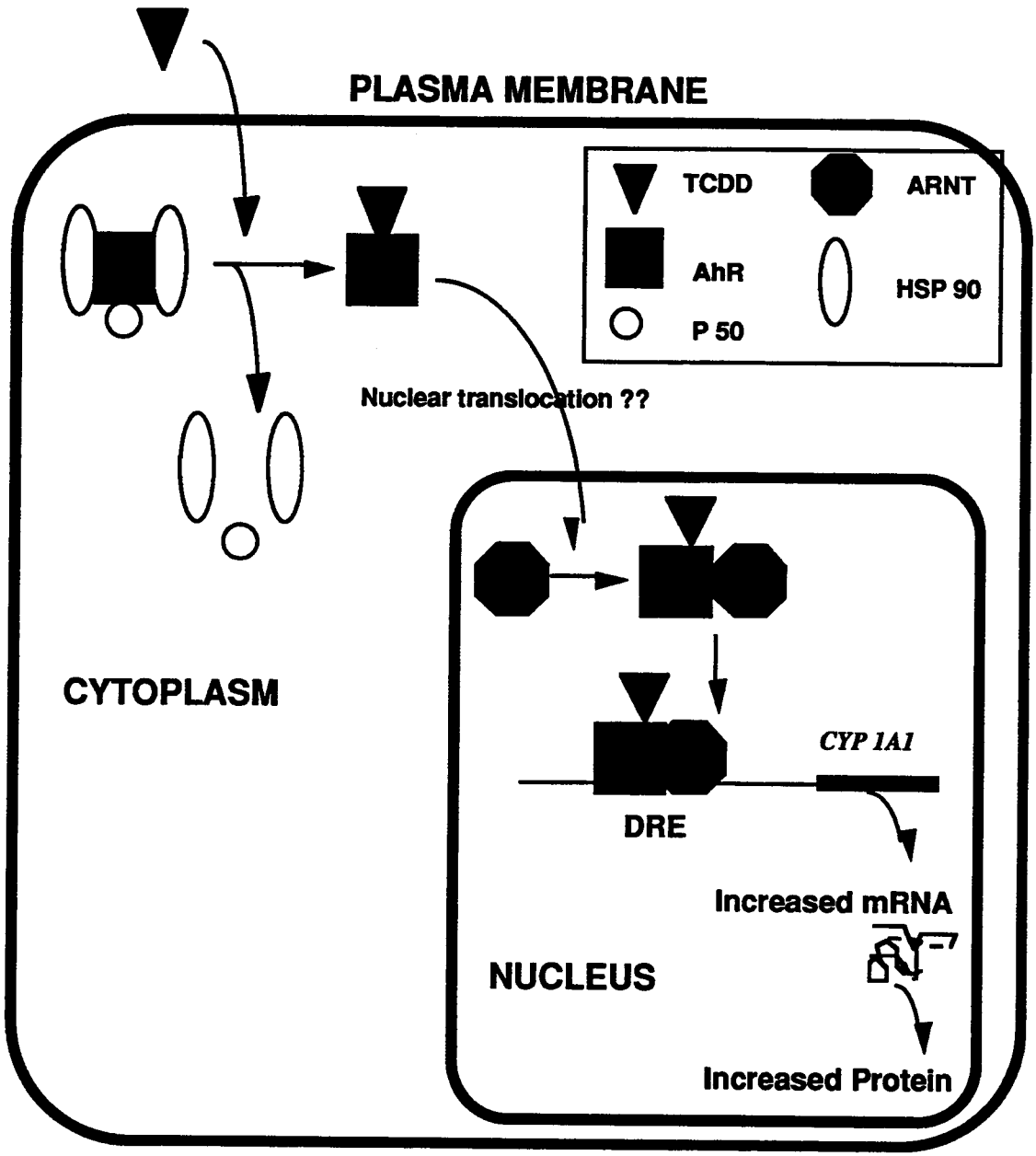

PLASMA MEMBRANE
TCDD
ARNT
AhR
HSP 90
P 50
Nuclear translocation ??
CYTOPLASM
CYP 1A1
DRE
Increased mRNA
NUCLEUS
Increased Protein

Figure 1.2

genetic backgrounds which are known to differ at the Ah locus (Vecchi *et al.* 1983). Congenic mice(C57BL/6J $Ah^{d/d}$) have the same genetic background as C57BL/6J $Ah^{b/b}$ mice except for the Ah locus (Poland *et al.* 1989). Congenic mice produce a lower TCDD binding affinity Ah receptor termed $Ah^{d/d}$ relative to $Ah^{b/b}$ which has a high binding affinity for the TCDD. C57BL/6J are characterized by the $Ah^{b/b}$ high affinity receptor. Comparative studies between these two mouse streans show that a higher dose of TCDD is required in $Ah^{d/d}$ mice to induce same intensity of toxicity as in $Ah^{b/b}$ mice (Poland and Glover 1980; Birnbaum *et al.* 1990).

CYP1A1 is not the only gene responsive to TCDD. There are some other genes directly responsive to TCDD exposure. CYP1A2 (Nebert and Gonzalez 1987), UDP-glucuronosyltransferase (Owens 1977), glutathione S-transferase Ya subunit (Rushmore and Pickett 1990), NAD(P)H:quinone oxidoreductase (Jaiswal 1991), and aldehyde dehydrogenase (Dunn *et al* 1988;Takimoto *et al.* 1991) are under direct transcriptional regulation by Ah receptor. Sparrow and his colleagues recently reported that PC levels and activity are suppressed by TCDD in C57BL/6J male mice (Sparrow *et al.* 1994). In chapter 2 of this thesis, experimental results are presented that implicate the Ah receptor involvement in the mediation of PC levels and activity. Congenic C57BL/6J $Ah^{d/d}$ male mice exposed to TCDD were employed in this investigation. It is further shown that suppression of PC takes place at the transcriptional level of the PC gene expression. Because TCDD liganded Ah receptor acts as a transcription factor, it is necessary to investigate the receptor's interaction with the PC gene regulatory regions. Cloning of regulatory regions in mice and characterization of the responsiveness of the PC gene to to TCDD exposure in mouse hepa1c1c7 cell culture system are described in chapter 3.

In summary, this thesis describes the possible mechanism of action of TCDD on the down-regulation of PC at the molecular level. Here I show Ah receptor mediation in reduced PC gene expression and responsiveness of cloned regulatory regions of the PC gene to TCDD exposure.

1.2 References

Barney, R. S., Cynthia, T. S., Ryu, B. -W., Selevonchick, D. P., and Schaup, H. W. (1994). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced alteration of pyruvate carboxylase level and lactate dehydrogenase isozyme shifts in C57BL/6J male mice. *J. Biochem. Toxicol.* **9:** 329-335.

Barsotti, D.A., Abrahamson, L.J. and Allen, J.R. (1979). Hormonal alterations in female rhesus monkeys fed a diet containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *Bull. Environ. Contam. Toxicol.* **211:** 463-469

Birnbaum, L. (1986). Distribution and excretion of 2,3,7,8-tetrachlorodibenzo-p-dioxin in congenic strains of mice which differ at the Ah locus. *Drug Metabol. Dispos.* **14:** 34-40.

Birnbaum, L. (1991). Developmental toxicity of TCDD and related compounds: Species sensitivities and differences. *Banhury Report* **35:** 51-68.

Birnbaum, L.S., McDonald, M.M., Blair, P.C., Clark, A.M., and Harris, M.W., (1990). Differential toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J mice congenic at the Ah Locus. *Fundam. Appl. Toxicol.* **15:** 186-200.

Burbach, K. M., Poland, A., and Bradfield, C. A., (1992). Cloning of Ah receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89:** 8185-8189.

Chahoud, I, Krowke, R., Schimmel, A., Merker, H., and Nebert, D. (1989). Reproductive toxicity and pharmacokinetics of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, I. Effects of high doses on the fertility of male rats. *Arch. Toxicol.* **63:** 432-439.

Couture, L.A., Abbott, B.D., and Birnbaum, L.S. (1990). A critical review of the developmental toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Recent advances toward understanding the mechanism. *Teratology* **42:** 619-632.

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

DeVito, M. J., and Birnbaum, L. S. (1994). Toxicology of Dioxin and related chemicals. *Dioxin and Health*. Schecter A., Plenum Press: 139-162.

Dunn, T.J., Lindahl, R., and Pitot, H.C. (1988). Differential gene expression in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): noncoordinate regulation of a TCDD-induced aldehyde dehydrogenase and cytochrome P-450c in the rat. *J. Biol. Chem.* **263:** 10878-10886.

Evans, R., (1988). The steroid and thyroid hormone superfamily. *Science* **240:** 889-892.

Fingerhut, M.A., Halperin, W.E., Marlow, D.A., Piacitelli, L.A., Honchar, P.A., Sweeney, M.H., Griele, A.L., Dill, P.A., Steenland, K., and Suruda, A.H. (1991). Cancer mortality in workers exposed to 2, 3, 7, 8 -tetrachlorodibenzo-*p*-dioxin. *N. Engl. J. Med.* **324:** 212-218.

Henck, J. W., New, M. A., Kociba, R. J., and Rao, K. S. (1981). 2,3,7,8-tetrachlorodibenzo-p-dioxin: Acute oral toxicity on hamsters. *Toxicol. Appl. Pharmacol.* **59:** 405-407.

Hoffman, E. C., Reues, H., Chu, F. F., Sander, F.,Conley, N. H.,Brooks, B. A., and Hankinson, O., (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252:** 954-958.

Jaiswal, A.K. (1991). Human NAD(P)H: quinone oxidoreductase (NQQ_1) gene structure and induction by dioxin. *Biochemistry* **30:** 10647-10653.

Jones, M.K., Weisenburger, W.P., Sipes, I.G.and Russel, D.H. (1987). Circadian alterations in prolactin, corticosterone, and thyroid hormone levels and down-regulation of prolactin receptor activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* **87:** 337-350.

Kerkvliet, N. (1994). Immunotoxicology of Dioxin and Related Chemicals. *Dioxin and Health.* Schecter A.,Plenum Press: 139-162.

Kociba, R.J., Keller, P.A., Park, C.N. and Gehring, P.J. (1976). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD): Results of a 13-week oral toxicity study in rats. *Toxciol. Appl. Pharmacol.* **35:** 553-574.

Lentnek, M., Griffith, O.W., and Rifkind, A.B. (1991). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin increases reliance on fats as a fuel source independently of diet: Evidence that diminished carbohydrate supply contributes to dioxin lethality. *Biochem. Biophy. Res. Comm.* **174:** 1267-1271.

Mackay, D., Shiu, W.-Y., and Ma, K. (1992). *Illustrated hand book of Physical -chemical properties and Environmental Fate of Organic chemicals Volume II*, Lewis Publishers, Chelsea. MI.

Martin, G., Vincent, N., Combet, C., and Baverel, G. (1993). A simple model for alanine metabolism in isolated rat hepatocytes. *Biochem. Biophys. Acta.* **1175:** 161-173.

Max, S.R., and Silberlgeld, E.K. (1987). Skeletal muscle glucocorticoid receptor and glutamine synthetase activity in the wasting syndrome in rats treated with 2, 3, 7, 8 -tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmcol.* **87:** 523-527.

McConnell, E.E. and Moore, J.A. (1979). Toxicopathology characteristics of the halogenated aromatics. *Ann. N.Y. Acad. Sci.* **320:** 138-150.

Mocarelli, P., Marocchi, A., Brambilla, P., Gerthoux, P.M., Colombo, L., Mondonico, A., and Meazza, L. (1991). Effects of dioxin exposure in humans at Seveso, Italy. *Banbury Report* **35:** 95-100.

Moore, R.W., Parsons, J.A., Bookstaff, R.C. and Peterson, R.E. (1985). Androgenic deficiency in male rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* **79:** 99-111.

Nebert, D.W., and Gonzalez, F.J. (1987). P450 genes: structure, evolution, and regulation. *Annu. Rev. Biochem.* **56:** 945-993.

Olson, J. L., Holscher, M. A., and Neal, R. A. (1980). Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the Golden Syrian Hamster. *Toxicol. Appl. Pharmacol.* **55:** 67-78.

Owens, I.S. (1977). Genetic regulation of UDP-glucuronosyltransferase induction by polycyclic aromatic compounds in mice: co-segregation with arylhydrocarbon hydroxylase induction. *J. Biol. Chem.* **252:** 2827-2833.

Perdew, G. (1990). Chemical cross-linking of the cytosolic and nuclear forms of the Ah receptor in hepatoma cell line 1c1c7. *Biochem. Biophys. Res. commun.* **182:** 55-62.

Peterson, R.E., Seefeld, M.D., Christian, B.J., Potter, C.L., Kellin, C.K., and Keesey, R.E. (1984). The wasting syndrome in 2, 3, 7, 8 -tetrachlorodibenzo-p-dioxin toxicity: Basic features and their interpretation. *Banbury Report* **18:** 291-308.

Pirkle, J. L., Wolfe, W. F., and Patterson, D. G. (1989) Estimate of half-life of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Vietnam veterans of Operation Ranch Hand. *J. Toxicol. Environ. Health* **27:** 165-171.

Pitot, H.C., Goldsworthy, T., Campbell, H.A., and Poland, A. (1980). Quantitative evaluation of the promotion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxins of hepatocarcinogenesis from dietylnitrosamine. *Cancer Res.* **40:** 3616-3620.

Poland, A. and Glover, E. (1980). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Segregation of toxicity with the Ah locus. *Mol. Pharmacol.* **17:** 86-94.

Poland, A., and Knutson, J. C. (1982). 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Ann. Rev. Pharmacol. Toxicol.* **22:** 517-524.

Poland, A., Teitelbaum, P., Glover, E., and Kende, A. (1989). Stimulation of *in vivo* hepatic uptake and in vitro binding of [^{125}I]2-Iodo-3,7,8-tetrachlorodibenzo-*p*-dioxin by the administration of agonists for the Ah receptor. *Mol. Pharmacol.* **36:** 121-127.

Reyes, H., Reisz-Porszasz, S., and Hankinson, O. (1992). Identification of Ah receptor nuclear translator protein (ARNT) as a component of the DNA binding form of the Ah receptor. *Sciences* **256:** 1193-1195.

Robinson, B.H., Oei, J., Sherwood, W.G., Applegarth, D., Wong, L. Haworth, J., Goodyer,P. Casey, R. and Zaleski, L.A. (1984). The molecular basis for the two different clinical presentations of classical pyruvate carboxylase deficiency. *Am. J. Human Gen.* **36:** 283-252.

Rushmore, T.H., and Pickett, C.B. (1990). Transcriptional regulation of the rat glutathione *S*-transferase Ya subunit gene: characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J. Biol. Chem.* **265:** 14648-14653.

Rutledge, S.L., Snead, O.C., Kelly, D.R., Kerr, D.S., Swann, J.W., Spink, D.L., and Martin, D.L. (1989). Pyruvate carboxylase deficiency: Acute exacerbation after ACTH treatment of infantile spasm. *Pediatr. Neurol.* **5:** 249-252.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds; Environmental and mechanistic considerations which support the development the toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* **21:** 219-238.

Scheuplein, R. J., Gallo, M. A., and van der Heijden, D. A. (1991). Epilogue, *Banbury Report* **35:** 489-490.

Schwertz, B. A., Norris, J. M., Sparschu G. L., Rowe, V. K., Gehring, P. J., Emerson, J. L., and Gerbig, C. G. (1973). Toxicology of chlorinated dibenzo-p-dioxins. *Environ. Health Perspect.* **5:** 87-99.

Stahl, B.U., Beer, D.G., Weber, L.W.D. and Rozman, K. (1993). Reduction of hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is due to decreased mRNA levels. *Toxicology.* **79:** 81-95.

Takimoto, R., Lindahl, R., and Pitot, H.C. (1991) *Superinduction of 2,3,7,8-tetrachlorodibenzo-p-dioxin- inducible expression of aldehyde* dehydrogenase by the inhibition of protein synthesis. *Biochem. Biophys. Res. Commun.* **180:** 953-959.

Turteltaub, K.W., Felton, J.S., Gledhill, B.L., Vogel, J.S., Southon, J.R., Caffee, M.W., Finkel, R.C., Nelson, D.E., Procter, I.D., and David, J.C. (1990). Accelerator mass spectrometry in biomedical dosimetry: Relationship between low-level exposure and covalent binding of heterocyclic amine carcinogens to DNA. *Proc. Natl. Acad. Sci. USA* **87:** 5288-5292.

Umbreit, T.H., Hesse, E.J., Macdonald, G.J. and Gallo, M.A. (1987). Effects of TCDD-estradiol interactions in three strains of mice. *Toxicol. Lett.* **40:** 1-9.

Vecchi, A., Sironi, M., Canegreti, M. A., Recchis, M., and Garattini, S. (1983). Immunosuppressive effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in strain of mice with different susceptible to induction aryl hydrocarbon hydroxylase. *Toxicol. Appl. Pharmacol.* **68:** 434-441.

Vos, J.G., and Moore, J.A. (1974). Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxins. Int. Arch. *Allergy Appl. Immunol.* **47:** 777-791.

Vos, J.G., Moore, J.A. and Zinkl, J.G. (1973). Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system of laboratory animals. *Environ. Health Perspect.* **5:** 149-162.

Vos, J.G., Van Loveren, H. and Schuurman, H.-J. (1991). Immunotoxicology of dioxin: Immune function and host resistance in laboratory animals and humans. *Banbury Report* **35:** 79-93.

Wassom, J.S., Huff, J.E. and Lortriano, N.A. (1977). A review of the genetic toxicology of chlorinated dibenzo-p-dioxins. *Mutat. Res.* **47:** 141-160.

Weber, L.W.D., Greim, H., and Rozman, K. (1987). Metabolism and distribution of [^{14}C] glucose in rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *J. Toxicol. Environ. Health.* **22:** 195-206.

Weber, L.W.D., Lebofsky, M., Stahl, B.U., Kettrup, A., and Rozman, K. (1992). Comparative toxicity of four chlorinated dibenzo-p-dioxins (CDDs) and their mixture. Part II: Structure-activity relationships with inhibition of hepatic phosphoenolpyruvate carboxykinase, pyruvate carboxylase, and glutamyl transpeptidase activities. *Arch. Toxicol.* **66:** 478-483.

Whitlock, J. P. (1990). Genetic and molecular aspect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* **30:** 251-277.

Zinkl, J.G., Vos, J.G., Moore, J.A., and Gupta, B.N. (1973). Hematological and clinical chemistry effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals. *Environ. Health Perspect.* **5:** 111-118.

Chapter 2

Ah Receptor Involvement in Mediation of Pyruvate Carboxylase Levels and Activity in Mice Given 2,3,7,8-Tetrachlorodibenzo-p-dioxin.

Byung-Woo Ryu and Henry W. Schaup

2.1. Abstract

The aryl hydrocarbon receptor (AhR) plays a central role in mediating 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity in animals. The investigations described here provide evidence that support a role for the AhR in TCDD-mediated pyruvate carboxylase (PC) level/activity reductions in mice. Pyruvate carboxylase plays a pivotal role in gluconeogenesis and in supplying carbon units for the citric acid cycle. Delivered i.p. in a corn oil carrier, TCDD suppresses PC activity/amount at doses as low as 1 μg/kg in responsive C57BL/6J ($Ah^{b/b}$) mice. Corn oil alone injected ip into mice at 4 ml/kg appears to be an inducer that increases the amount and activity of PC. However, TCDD suppresses this induction. In the $Ah^{b/b}$ mouse, PC levels and activity are reduced to 10% control values at a TCDD dose of 75 μg/kg. A time-course experiment shows that the PC reductions are apparent within 16 hours post-TCDD exposure. Here we report investigations on the PC/TCDD response using a congenic C57BL/6J ($Ah^{d/d}$) mouse strain having an AhR with a low affinity for TCDD. If the PC/TCDD response is AhR mediated, the congenic mouse strain ($Ah^{d/d}$) would require much higher doses of TCDD to suppress PC. In the $Ah^{d/d}$ mice, we observe that an approximately 60-fold increase in TCDD dose is necessary to produce a PC/TCDD effect. The different response in PC mRNA reduction caused by TCDD exposure between the $Ah^{b/b}$ and $Ah^{d/d}$ mice is obserbed. We also find that in $Ah^{d/d}$ mice, the corn oil carrier does not appear to induce an increase in PC activity/amounts, as reported for $Ah^{b/b}$ mice.

2.2. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) produces an extensive range of effects in animals that vary with both tissue and species (Whitlock,

Jr. 1993). Alterations in metabolic pathways in the liver represent one of the hallmarks of TCDD exposure (Poland and Knutson 1982). The molecular mechanisms by which TCDD precipitates a series of metabolic failures are not fully understood. However, it is generally accepted that the aryl hydrocarbon receptor (AhR) plays an essential role in mediating or initiating all deleterious effects of TCDD exposure (Whitlock, Jr. 1993; Whitlock, Jr. 1990). The best mechanistic characterization of AhR activity is that described for it enhancement of CYP1A1 gene transcription (Israel and Whitlock, Jr. 1983; Israel and Whitlock, Jr. 1984). Aryl hydrocarbon receptor involvement in down regulation of transcription or protein synthesis remains to be fully characterized.

In a previous report, it was proposed that TCDD mediates reductions in pyruvate carboxylase (PC) amount/activity and that this response represents a key component in TCDD toxicity in C57BL/6j ($Ah^{b/b}$) male mice (Sparrow *et al.* 1994). A TCDD dose-dependent shift in lactate dehydrogenase isozyme patterns coupled with an increase in plasma lactate level are also reported. This isozyme shift was observable at doses that were greater than required for a reduction in PC. Pyruvate (PCs substrate) plays a pivotal role in the citric acid cycle and gluconeogenesis. Therefore, the mechanism of action of TCDD that produces a reduction of PC and, hence, a reduction in pyruvate utilization, is of importance if we are to understand the impact of TCDD on intermediary metabolism.

Here we have used a C57BL/6j ($Ah^{d/d}$) congenic mouse strain to implicate AhR involvement with PC down-regulation in mice. Congenic strains harbor only a small fraction of the donor strain's genetic background (Martin *et al.* 1992; Demant and Hart 1986). This enables one to interpret results with minimal concern for background genetic variation and ascribe

results to the target locus, which is the AhR locus in this case. The AhR locus in the congenic strain employed here produces an AhR ($Ah^{d/d}$) that manifests a much lower affinity for TCDD than the normal C57BL/6j ($Ah^{b/b}$) locus gene product (Poland and Glover 1980; Okey *et al.* 1989; Silkworth *et al.* 1989). Accordingly, an appreciably higher dose (4- to 20- fold) is necessary to produce a TCDD response in the congenic strain. Characteristic responses to TCDD, such as thymic involution and cleft palate, segregate with the AhR locus such that in congenic strains, large TCDD doses (relative to that required for the ($Ah^{b/b}$) mice) are necessary to elicit the toxic response. This dose response provides prima facie evidence for AhR involvement in mediating the TCDD response (Poland and Glover 1980; Jones and Sweeney 1980; Knutson and Poland 1982).

In this investigation, we found that a reduction in PC level/activity and PC mRNA level in ($Ah^{d/d}$) mouse hepatic tissue requires higher doses of TCDD than required for ($Ah^{b/b}$) mice. Presumably, this is a consequence of the lower binding affinity of the $Ah^{d/d}$ receptor for TCDD. Lactate dehydrogenase (LDH) isozyme and plasma lactate level did not change over the dose range investigated. Previously reported corn oil-induced increases in level and activity of PC in $Ah^{b/b}$ mice were not observed in $Ah^{d/d}$ mice used in this study (Sparrow *et al.* 1994).

2.3. Experimental Procedures

2.3.1 Reagents

Crystallized TCDD (99% pure) was a generous gift from Dr. S.Safe (Texas A&M University, College Station, TX). Coenzyme A, ATP. citrate synthase (EC 4.1.3.7), citrate lyase (EC 4.1.3.6), lactate dehydrogenase (EC 1.1.1.27), phopsphotransacetylase (EC 2.3.1.8), malate dehydrogenase

(EC 1.1.1.37), acetylphosphate (K-Li salt), NADH, and pyruvate were purchased from Boehringer Mannheim (Indianapolis, IN). 1-Methyl-2-pyrrolidinone, 4-chloro-1-naphthol, Tween 20, lactate standard, and lactate kit were obtained from Sigma (St. Louis, MO). Zeta Probe membrane, avidin conjugate, biotinylated standard, and polyacrylamide (99% pure) were obtained from BioRad (Richmond, CA). All other chemicals were of highest quality grade possible.

2.3.2 Animals

Eight breeding pairs of congenic C57BL/6j ($Ah^{d/d}$) mice were a gift from Dr. Alan Poland (University of Wisconsin, Madison, WI). This congenic mouse strain was bred by backcross/intercross breeding between C57BL/6J and B6N.D2N (Ah^{d}) $N_{13}F_{13}$ originally bred by Dr. Daniel Nebert, National Institute of Child Health and Human Development (Bethesda, MD). This breeding produces congenic mice [B6N.D2N (Ah^{d}) $N_{13}F_{13}$.$B6JN_{28}F_{28}F_{3}$], which is homozygous for the $Ah^{d/d}$ allele (Poland *et al.* 1989). These mice were used to establish a colony that served as our source for this mouse strain. The mice were maintained in a room at a constant temperature (22 ± 1°C), humidity (40-60%), and lighting cycle 12 hours on/off). The room was maintained as a clean room with double entry. Shoe coverings, lab coats, and masks were required for admittance. Animal cages were positioned on a rack facing a laminar flow system. All animals were fed a standard laboratory stock chow (Harland Tekland, Madison, WI). Eight-week-old male mice weighing 22-24 g at the time of injection were held in individual cages and divided into three groups. Pair-fed control animals were given a fixed amount of food based on experimental animal food consumption. Experimental animals were given 1, 20, 50, 75 and 100 mg TCDD/ kg body

weight. One group of animals, those fed ad libitum, were not given TCDD or corn oil. For the time-course experiment, after 3 days of ad libitum feeding, animals were separately by weight and divided into four groups of six. Three were pair fed based upon experimental animal feed consumption, and three animals from each group were injected ip with 50 µg TCDD/kg body weight. Pair-fed animals were ip injected with 4 ml corn oil/ kg body weight. Animal weight and food consumption were monitored daily for all groups. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was prepared from a TCDD stock solution (0.102 mg/ml) in corn oil. The concentration of the TCDD stock solution was confirmed by mass spectrometry (OSU Environmental Health Sciences Center MS facilities). Pair-fed controls were injected ip with corn oil only. Body weight and food consumption of all animals were recorded daily.

2.3.3 Protein Extraction

Eight days post-TCDD administration, mice were sacrificed by CO_2 asphyxiation, and blood was collected by cardiac puncture for the lactate assay. The livers were excised, and the gall bladder was removed. Livers were washed with 0.9% saline, weighed, perfused with 0.9% ice-cold saline, and minced with a razor blade. The tissue homogenization was done in ice-cold homogenization buffer (1:3, w/v,pH 7.4) containing 20 mM Tris-HCl, 0.3 M manitol, 2 mM EDTA, 1 mM dithiothreitol, and 0.2 mM 4-(2-aminoethyl)-benzo sulfonyl fluoride using a 30 mL Potter-Elvehjem tissue homogenizer. Homogenates were fractionated at 29,000 x g for 10 minutes at 4°C. The supernatant was further fractionated by centrifugation at 368,000 x g for 45 minutes at 4°C. The aqueous supernatant fraction was concentrated with an Amicon (Beverly, MA) centriprep concentrator (30 kD exclusion limit). Glycerol was added to the retantate to bring it to 10% glycerol. These

preparations were divided into small portions and stored at -85°C until use. Protein concentrations were determined by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976).

2.3.4 Pyruvate Carboxylase Assay

The method of Berndit *et al.* was employed for the PC activity assay (Berndit *et al.* 1978). The citrate-forming reaction via PC (0.15 mg/600 µl) was started by addition of tissue extracts to a reaction mixture containing 100 µmol Tris-HCl (pH 8.0), 2 µmol pyruvate, 1 µmol ATP, 5 µmol acetylphosphate, 0.5 µmol CoASH, 5 µmol $MgCl_2$, 15 µmol $KHCO_3$, 2 U citrate synthase, and 10 U phopsphotransacetylase. As a control, PC was inactivated by avidin (1 U), which was added to the tissue extract before incubation. After 30 minutes incubation at 37°C, the reaction was stopped by the addition of 15 mg KBH_4. The amount of citrate produced, which is proportional to PC activity, was determined by the method of Mollering (Mollering 1985).

2.3.5 Western Blotting

Protein from liver extracts (between 90 and 120 µg protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Hames and Rickwood (Hames and Rickwood 1990). For western blotting, the gel was equilibrated with 200 ml of blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3) for 25 minutes to remove excess SDS. Zeta probe membrane was soaked with blotting buffer for 15 minutes. Transfer of separated protein onto Zeta-probe membrane was accomplished using a Genie Blotter (Idea Scientific, Minneapolis, MN). To develop a blot, the membrane was incubated and conjugated with avidin peroxidase, as previously described by McKim *et al.* (McKim *et al.* 1991).

Because pyruvate carboxylase has biotin covalently bound, the avidin peroxidase alone is sufficient to complete the visualization step. The blots produced from time-course experiments were scanned using a Molecular Dynamics scanning system and associated software. The theoretical PC reductions for a half-life of 110.4 hours were calculated using the following decay equation: $N = N_0 e^{-\lambda t}$, where N_0 is the control pixel density at t = 2,4,16, or 192 hours. The decay constant is calculated from $l = 0.693/T_{1/2}$. This curve is showed later in Figure 4, where time is plotted as log hours for convenience.

2.3.6 Preparation of ^{32}P-Labeled Oligonucleotide Probe

To analyze mRNA levels of pyruvate carboxylase, a 39-base oligonucleotide probe designated MPC-1 was synthesized chemically by our Center for Gene Research and Biotechnology, Oregon State University. The probe sequence: 5' CAT GTC CTT GGT AAC ATG AAC CTT TCG GAT AGT GCC CTC-3 is complementary to nucleotides 3560 to 3598 of mouse pyruvate carboxylase (Zhang *et al.* 1993). This sequence was utilized because it is unique to PC as compared to other biotin-containing enzymes (e.g., acetyl CoA carboxylase, 3 Me carbonyl CoA carboxylase, and propionyl CoA carboxylase). A Gen Bank database search revealed no significant homology between this sequence and known sequences coding biotin containing enzyme.

Another 15-base oligonucleotide probe designated 1406R was utilized to determine the abundance of the 18S ribosomal RNA for normalization of the RNA load on membrane blots. The probe sequence: 5' ACG GGC GGT GTG TRC 3' is complementary to nucleotides 1406 to 1392 of *Escherichia coli* 16S rRNA. This is a universal sequence found in all

prokaryotic and eukaryotic organism's small subunit rRNAs (Pace *et al.* 1988).

After chemical synthesis was completed, both probes were purified using C-18 Sep-Pak cartridges. Probes were radiolabeled to specific activity (~5 X 10^8 dpm/ mg) with γ^{32}P-ATP using a 5' end-labeling method (Sgaramella and Khorana 1972). The oligonucleotide was separated from free γ^{32}P-ATP using P2 gel chromatography.

2.3.7 Cellular RNA Preparation and Northern Analysis

Total RNA was extracted from liver samples using guanidiumthiocyanate-phenol-chloroform according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Liver samples were homogenized with a polytron at 1/2 maximum speed for 10 seconds while placed on ice. RNA quantitation and purity were determined spectrophotometrically using the A_{260} and the 260/280 nm of the sample. Samples of 50 µg of total RNA were electrophoresed on 2.2 M formaldehyde-containing agarose gels. The quality of extracted RNA samples was checked by ethidium bromide staining of agarose gels to verify the 28S and 18S bands and their intensity. Gels were washed overnight in excess doubly distilled H_2O to remove formaldehyde and then were transferred overnight to a Zeta-probe membrane (Bio-Rad). RNA was immobilized by baking the filters for 1 hour under vacuum at 80°C. Baked membranes were prehybridized for 1 hour at 55°C in hybridization buffer (0.5 x SSC, 3.5% SDS, 125 mM phosphate buffer). Following prehybridization, the immobilized RNA was hybridized for 18 hours to ^{32}P-labeled oligonucleotide probes. Following hybridization, membranes were washed. Membranes were wrapped in Saran Wrap and exposed with

Dupont Cronex Plus intensifying screens for 0.5-4 days to Kodak X-Ompt film at -90°C. Quantitation of RNA signals was accomplished via scanning densitometry. A Molecular Dynamics (Sunnyvale, CA) scanning system and analytical software was employed for this purpose. The data from autoradiographs are reported as image density units using the software default settings.

2.3.8 Lactate Assay

Blood was collected into 3 ml Vacutainer tubes containing sodium fluoride and potassium oxalate (Becton Dickinson, Rutherford, NJ). The blood was obtained by cardiac puncture from pair-fed control and TCDD-treated mice (1,20,50,75 and 100 μg/kg dose). Each treatment group consisted of three mice. The plasma was separated immediately by centrifugation at 400 x g for 10 minutes at 22°C. Plasma lactate level was determined using a lactate kit (#735) and a lactate standard using a protocol obtained from Sigma (St.Louis, MO). The assays were done in triplicate.

2.4. Results

At 8 days postinjection, the ratios between mouse liver and body weights were 0.058 ± 0.003 for pair-fed control and averaged 0.064, 0.070, 0.072, 0.076 and 0.092 for each TCDD treatment group (1,20,50,75 and 100 μg/kg body weight). The mean ratio for TCDD treatment groups was 0.075 ± 0.005. Liver reticulation was apparent visually only in 75 and 100 μg/kg TCDD treatment groups. However, over the TCDD dose range, treatment groups displayed a dose-dependent hepatomegally when compared to pair-fed controls.

Hepatic PC activity relative to pair-fed controls was determined for animals given various amounts of TCDD (1-100 μg/kg body weight). Figure 2.1 shows the PC enzymatic activity profile. Significant differences in activity between control and experimental animals were not seen until the dose exceeded 50 μg/kg body weight). The reduction of PC activity seen here in $Ah^{d/d}$ mice is approximately 60 times greater than that found for $Ah^{b/b}$ mice (ED_{50} = ~ 1.0 μg/kg body weight) (Sparrow *et al.* 1994).

To confirm that changes in PC activity with respect to TCDD dose are accompanied by a reduction in PC mass, western blot analysis for PC in $Ah^{b/b}$ and $Ah^{d/d}$ mice was carried out. The results shown in Figure 2.2 are in agreement with those obtained in the activity assay. In these experiments, fixed amounts of total liver protein extract are compared. Pyruvate carboxylase level was reduced most obviously in $Ah^{b/b}$ mice with a single TCDD injection ip of 20 and 50 μg/kg body weight (lanes 6 and 8), as we have previously reported (Sparrow *et al.* 1994). However, TCDD has no suppressive effect at the same dose on PC level in $Ah^{d/d}$ mice (lanes 7 and 9). Therefore, PC reduction with TCDD exposure appears to be dependent on the AhR locus. It has observed that corn oil injection ip in $Ah^{b/b}$ mice appears to induce an increase in the PC amount. This is accompanied by an increase in the PC enzymatic activity by approximately fourfold (Sparrow *et al.* 1994). Figure 2.3 (lanes 2 and 3) shows this response comparing control $Ah^{b/b}$ animals not given corn oil with those given 4 mL/kg body weight. A similar comparison using $Ah^{d/d}$ mice reveals no difference between control and those given corn oil (lanes 4 and 5).

Figure 2.1 Hepatic pyruvate carboxylase enzyme activity. Enzyme activity in hepatic protein extracts from TCDD-treated $Ah^{d/d}$ mice were assayed as described in the experimental procedures section. Cross bars show the standard deviation for replicated samples using a minimum of six mice per dose

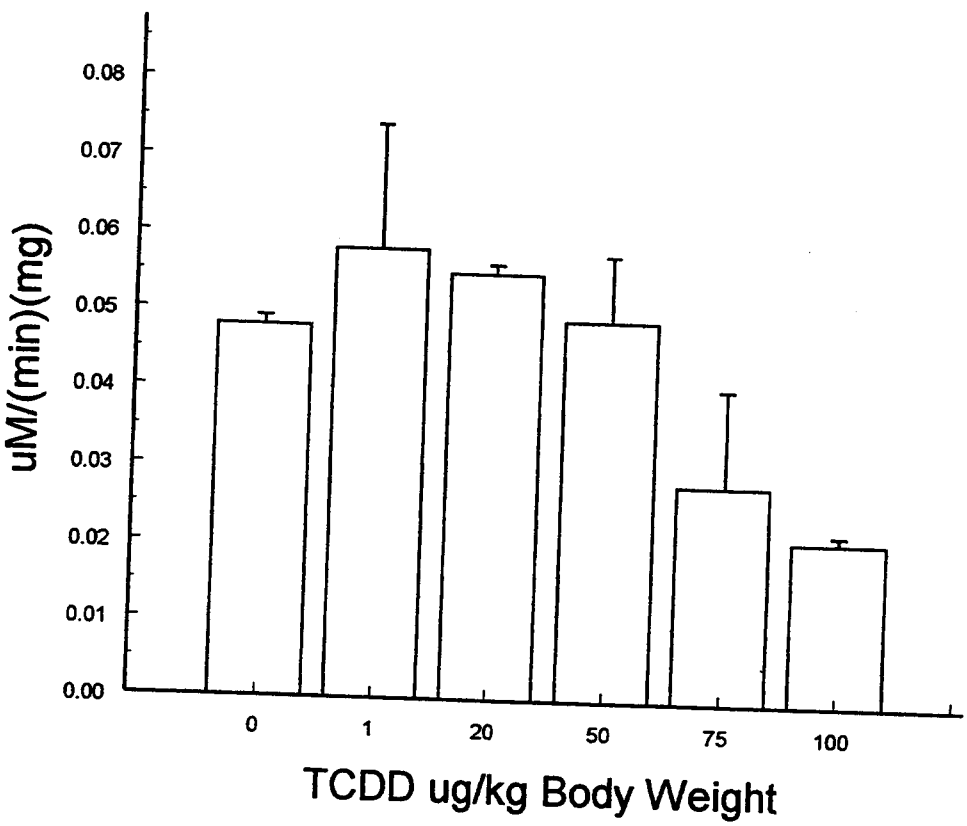

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
uM/(min)(mg)
0
1
20
50
75
100
TCDD ug/kg Body Weight

Figure 2.1

Figure 2.2 Western blot analysis of hepatic pyruvate carboxylase from $Ah^{b/b}$ and $Ah^{d/d}$ male mice. Lane 1 displays the following biotinylated standards: myosin (200,000 D), *E. coli* β-galactosidase (116,250 D), phosphorylase B (97,400 D), serum albumin (66,200 D), and ovalbumin (45,000 D). Lane 2 and 3 are samples from animals that fed ad libitum and pair-fed corn oil injected control mice, respectively. Lane 4,6, and 8 are samples from $Ah^{b/b}$ mice given 1, 20, and 50 μg TCDD/kg body weight, respectively. Lane 5,7, and 9 are samples from $Ah^{d/d}$ mice given 1, 20, and 50 μg TCDD/kg body weight, respectively. Protein (90 μg per lane) was loaded onto a 10 x10 x 0.08 cm SDS-polyacrylamide gel (3.5% stacking, 6% resolving) and separated at 150 V constant voltage for 1.8 h at anbient temperature. Protein was transferred onto Zeta-probe membrane at 5 V constant voltage at 4°C for 16 h.

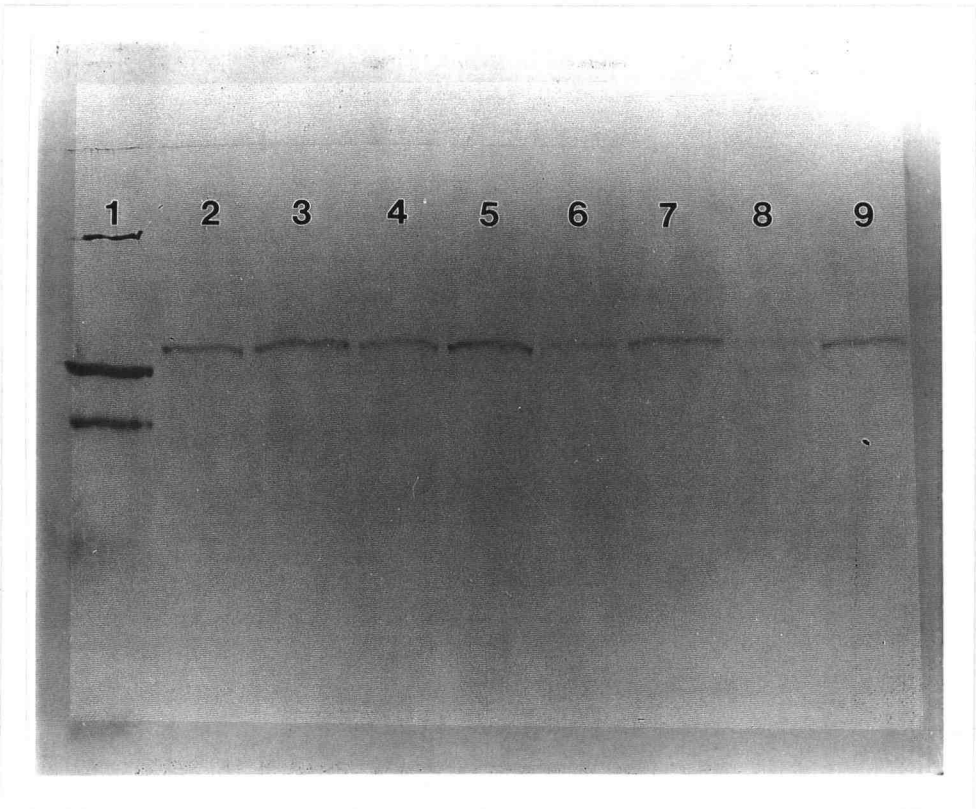


Figure 2.2

Figure 2.3 Western bolt analysis of hepatic pyruvate carboxylase from $Ah^{b/b}$ and $Ah^{d/d}$ male mice. Lane 1 is biotinylated standards: *E. coli* β-galactosidase (116,250 D), phosphorylase B (97,400 D). Lane 2 and 3 are samples from $Ah^{b/b}$ mice fed ad libitum and pair-fed corn oil injected controls, respectively. Lane 4 and 5 are samples from $Ah^{d/d}$ mice fed ad libitum and pair-fed corn oil injected controls, respectively. Protein (120 μg per lane) was loaded onto a 10 x10 x 0.08 cm SDS-polyacrylamide gel (3.5% stacking, 6% resolving) and separated at 150 V constant voltage for 1.8 h at anbient temperature. Protein was transferred onto Zeta-probe membrane at 5 V constant voltage at 4°C for 16 h.

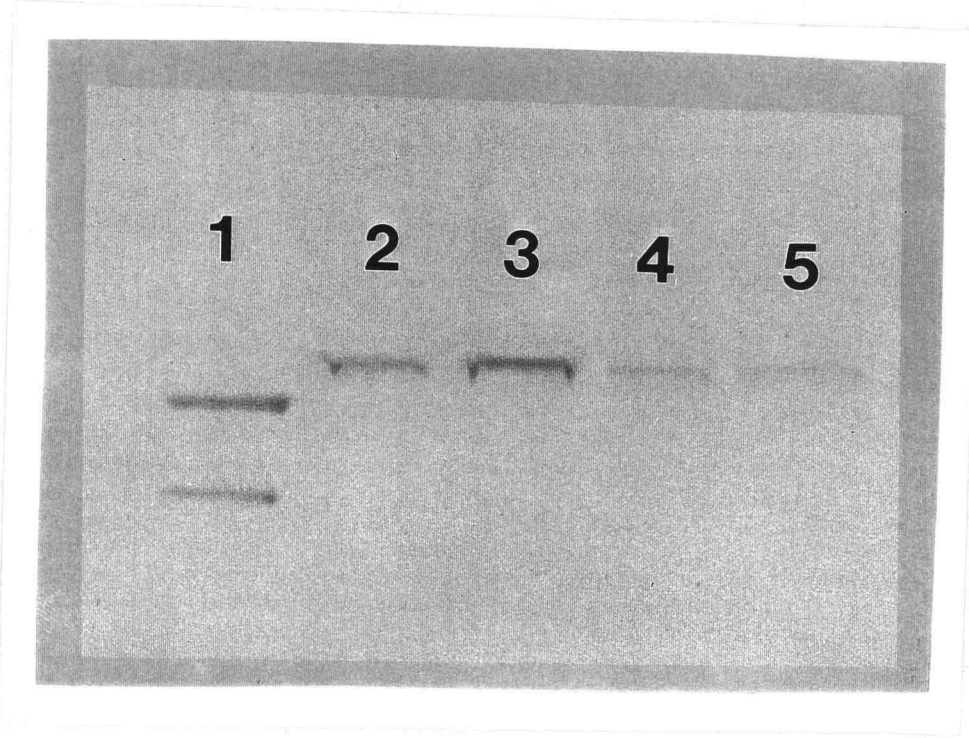


Figure 2.3

Previously, it was reported that PC activity/amount reduction appears to produce a dose-dependent increase in blood lactate level. An LDH isozyme pattern shift in $Ah^{b/b}$ mice was also reported within the dose range investigated here (Sparrow *et al.* 1994). Here, an LDH isozyme pattern shift was not observed in $Ah^{d/d}$ mice (data not shown), nor did blood plasma lactate level increase in the dose range of 1 μg to 100 μg TCDD/kg body weight (Figure2.4). This segregated dose response in blood plasma LDH levels by TCDD treatment between those two mice strains may be indirect evidence implicating the secondary effect of TCDD mediated by AhR in LDH reduction.

A time-course experiment using C57BL/6J $Ah^{b/b}$ mice reveals that the TCDD-induced reduction in PC levels in manifest between 4 and 16 hours post-treatment (Figure 2.5). The mice were given 50 μg TCDD ip/kg body weight. The reported half-life for PC is 110.4 hours in rats (Weinberg and Utter 1979). Therefore, if the primary disruptive event involves transcription or translation of PC mRNA, one would not expect to observe a large change in PC levels at early time points. The broken line in Figure 2.5 shows the expected reduction in PC levels if the half-life of the protein is 110.4 hours (rat PC half-life). The theoretical fit is consistent with what one would expect if synthesis of new PC were blocked by TCDD exposure.

The effect of exposure to various levels of TCDD in the two mouse strains $Ah^{b/b}$ and $Ah^{d/d}$ mice on hepatic PC mRNA is illustrated in Figure 2.6. Only 50 and 75 μg/kg body weight responses are shown for $Ah^{d/d}$ mice. In previous data (Figure 2.1), PC enzyme activity levels are not manifest at dose lower than 50 μg/kg body weight in $Ah^{d/d}$ mice. The abundance of the 18S mRNA was used for normalization of the RNA loaded on the membrane and

Figure 2.4 Plasma lactate dehydrogenase enzyme concentration. The enzyme levels in plasma from pair-fed control and TCDD treated $Ah^{d/d}$ mice were measured as described in the experimental procedures section. Cross bars show the standard deviation for replicate samples using a minimum of six mice per dose.

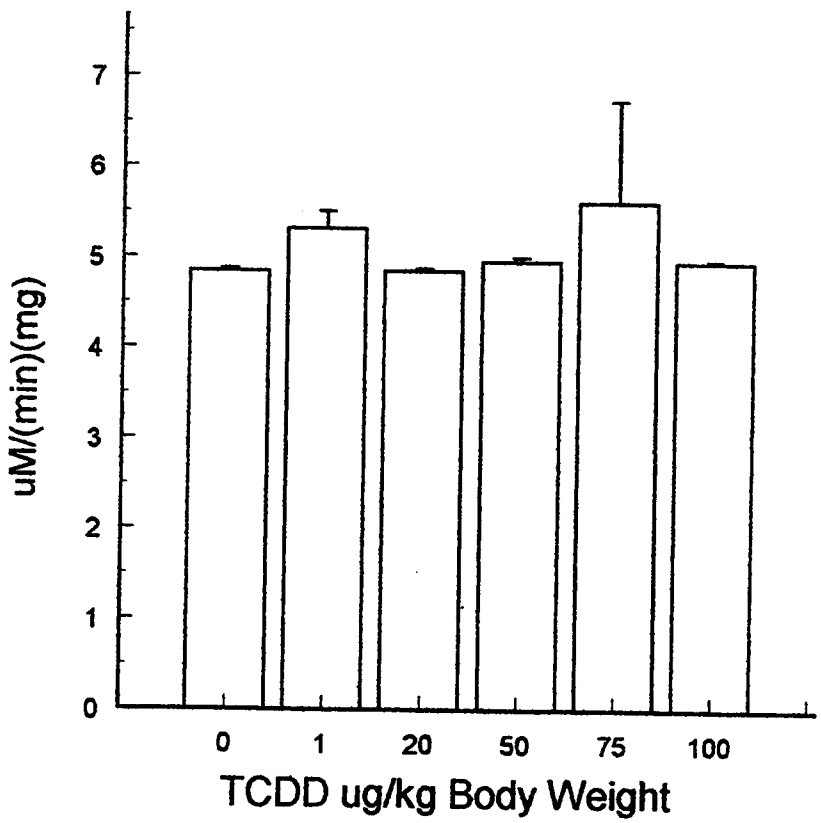


Figure 2.4

Figure 2.5 Time course analysis of TCDD-induced reduction on hepatic pyruvate carboxylase from western blots in C57BL/6J mice. Time 0 is an adlib control. Samples are from $Ah^{b/b}$ mice sacrificed at 2,4,18, and 192 h after exposure (i.p.) to 50 µg TCDD/kg body weight. Protein (50 µg) was loaded onto 10 x10 x 0.08 cm SDS polyacrylamide gels (3.5% stacking, 6% resolving gel) and separated at 200 V constant voltage for 1 h at ambient temperature. Proteins were transferred onto Zeta-probe membrane using 5 V constant voltage at 4°C for 15 h. Visualization was by avidin-peroxylase staining using 4-chloro-1-napthol. The blot was scanned, and pixel density was plotted (•). The dashed line (–•–) shows the theoretical loss of PC if a 110.4 h half-life is assumed (see text for detail).

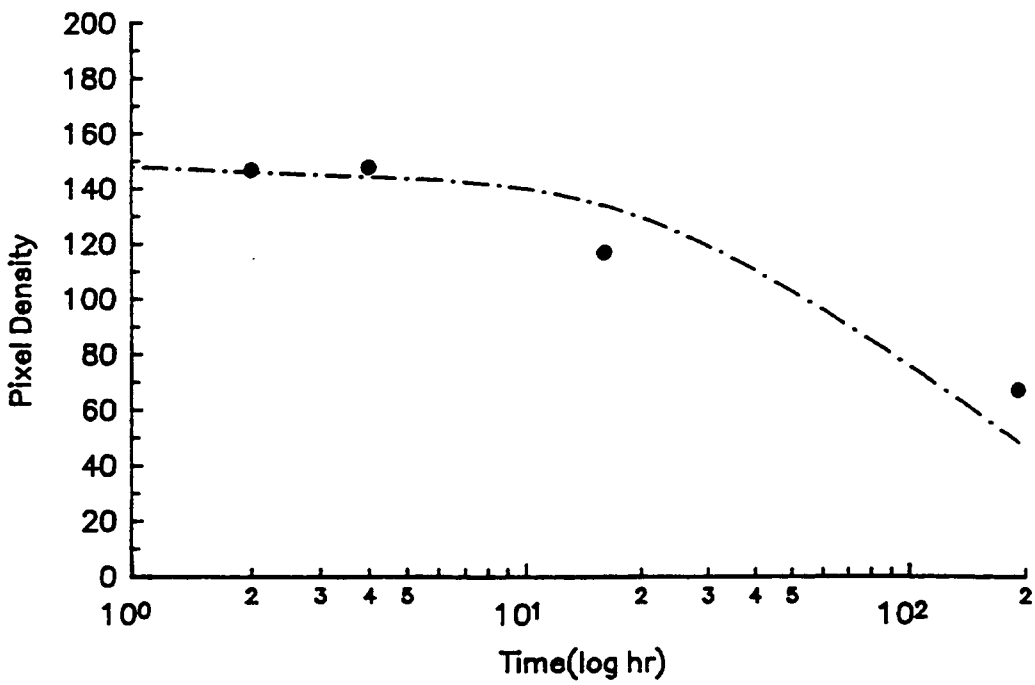

200
180
160
140
120
100
80
60
40
20
0
Pixel Density
10⁰
2
3
4
5
10¹
2
3
4
5
10²
2
Time(log hr)

Figure 2.5

Figure 2.6 Hepatic PC mRNA reductions in response to TCDD exposure. Animals were sacrificed 8 days post i.p. injection with TCDD. Northern blot analysis of RNA extracts from control and experimental animals was performed as described in materials and methods. The results shown are from an experiment where the number of animals per exposure was three. Autoradiographs of blots probed with PC mRNA probe, MPC-1, were scanned, and the ratio of image density control values to experimental values on the same blot were employed to produce the data for $Ah^{b/b}$ (•) and $Ah^{d/d}$ (♦) samples. The average for three independent determinations are shown. The R2 for $Ah^{b/b}$ data points regression is 0.793. Standard error for the ratios were calculated using error propagation analysis as described by (Benington 1969). The experiment with $Ah^{b/b}$ (•) mice are taken from Ilian, M. A., Sparrow, B. R., Ryu, B. -W., Selivonchick, D. P., and Schaup, H. W. (1996).

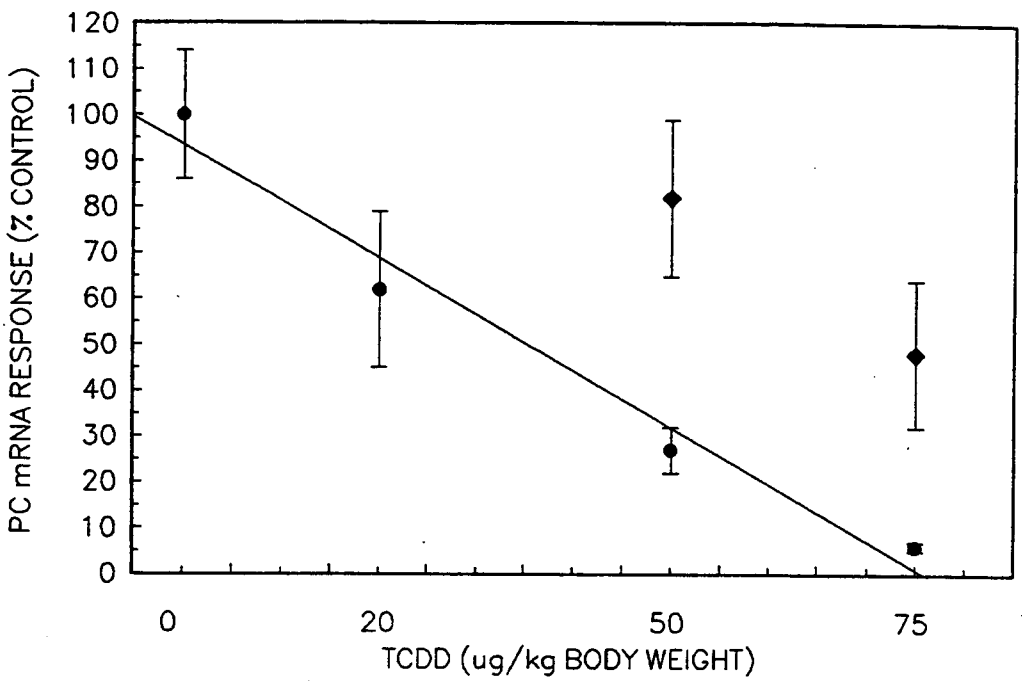

120
110
100
90
80
70
60
50
40
30
20
10
0
PC mRNA RESPONSE (% CONTROL)
0
20
50
75
TCDD (ug/kg BODY WEIGHT)

Figure 2.6

determined by direct hybridization using 1406 R probe. Exposure of animals to TCDD caused reduction in PC mRNA level in a dose dependent manner. At 50 and 75 μg/kg TCDD dose, the response of $Ah^{b/b}$ mice are respectively, 3- and 10-fold greater than that seen in $Ah^{d/d}$ mice. The reduction in PC mRNA levels is associated with a coincidental reduction of the PC level and enzyme activity. These results suggest that the effect of TCDD in mouse liver is at the transcriptional level.

2.5. Discussion

Inbred congenic animals are powerful experimental tools that can provide evidence for association of a response to a toxic substance with a specific genetic locus (Martin *et al.* 1992; Demant and Hart 1986). The congenic mice used in this work were derived through inbreeding DBA/2 mice, which have a "low" affinity $Ah^{d/d}$ receptor, with C57BL/6J mice, in which the receptor ($Ah^{b/b}$) displays a decidedly higher affinity for TCDD (Poland *et al.* 1989). These congenic mice have been used extensively in studies that focus on aromatic hydrocarbon toxicity (Poland *et al.* 1974; Poland and Glover 1979). The best molecular characterization of less responsive and responsive AhR effects are those associated with the transcription of the aryl hydrocarbon hydroxylase gene (*CYP1A1*) (Poland *et al.* 1974; Gonzalez *et al.* 1984). In this case, TCDD via an AhR interaction with *cis*-acting elements near the structural gene dramatically enhances transcription of *CYP1A1* mRNA. Many other genes are impacted by TCDD/AhR enhancement of transcription in a manner that genetically segregates with AhR responsiveness (Nebert and Gonzalez 1987; Nebert 1989). Here we report a comparable association between the suppression of PC levels/activity and the AhR locus. We base this conclusion on the

observation that it requires about a 60-fold larger dose of TCDD to suppress PC activity in $Ah^{d/d}$ mice relative to $Ah^{b/b}$ mice (Sparrow *et al.* 1994). Furthermore, the northern blot assay showing that PC mRNA reduction response to TCDD exposure in $Ah^{d/d}$ mice is in approximately 3-fold less sensitive than that in $Ah^{b/b}$ mice. This observation supports the conclusion that the response is prohibited by TCDD. This increased dose requirement is consistent with observations made for other genes in many investigations using responsive and nonresponsive AhR mice (Poland and Glover 1976; Kumaki *et al.* 1977; Owens 1977). The general conclusion, then, is that the PC response that we find requires a functional AhR.

It is the TCDD/AhR suppression of PC levels and activity that distinguishes our observations from those previously reported for other genes. No clear mechanism has emerged that would explain how the AhR might suppress transcription or translation or produce enhanced turnover of a protein. At this point, we expect the reductions we observe in PC $Ah^{b/b}$ mice to be the result of inhibition of PC mRNA transcription. This expectation is premised by the observation that the reduction in PC levels is observed with disruption of PC mRNA synthesis. The data in Figure 2.5 are in agreement with the reported half-life of PC (110.4 h) in rats (Weinberg and Utter 1979). We do not know if the dose employed produces a complete block in the synthesis of PC; however, the observed rate of PC loss supports this possibility. It does not seem likely that the effect is the result of enhanced turnover of the protein. If this were the case, we would expect a greater reduction in protein levels. For example, if the half-life were reduced to 55.2 hours (ie, increased turnover) we would not find detectable levels of PC in our experiments at the 192 hour points.

Further investigations are necessary to determine the molecular mechanism involved in AhR suppression of PC activity and levels in $Ah^{b/b}$ animals. Such investigations are warranted because of the central role PC plays in gluconeogenesis and the citric acid cycle (Weber *et al.* 1992; Weber *et al.* 1993). This enzyme is unique in as much as it functions in both catabolic and anabolic metabolism. Catabolically, reduction of PC with TCDD treatment prevents the entrance of pyruvate into the citric acid cycle, thus contributing to an intracellular energy imbalance. Pyruvate is also a substrate in gluconeogenesis. Treatment with TCDD causes hypoglycemia in animals as a consequence of a decrease in gluconeogenesis precipitated by the reduction of PC and phosphoenol-pyruvate carboxykinase (PEPCK). (Sparrow *et al.* 1994; Stahl *et al.* 1993). It has been proposed that reduced carbohydrate production caused by the TCDD effect on the intermediary metabolism may be a primary cause of animal lethality (Weber *et al.* 1991).

In a previous report, using $Ah^{b/b}$ mice, we noted that the corn oil vehicle for TCDD delivery by itself induces an increase in PC activity and amount relative to mice not given corn oil injection (Sparrow *et al.* 1994). We did not observe this induction in $Ah^{d/d}$ mice. The response Is interesting in light of recent reports describing the regulation of glutathione *S*-transferase, epoxide hydroxylase and UDP-glucoronosyltransferase by dietary lipids (Yang *et al.* 1993; Dannenberg *et al.* 1983; DeWille and Farmer 1993). It appears that lipids regulate levels of cytosolic as well as membrane-bound xenobiotic-metabolizing enzymes. If the congenic mice differ only with regard to the AhR locus, then our data are consistent with the possibility that the receptor may be a natural transcriptional regulator for

genes associated with energy metabolism. This possibility also warrants further investigations.

2.6 References

Benington, P. (1969). Data reduction and error analysis in the physical *sciences,* p. 64, McGraw Hill, New York.

Berndt, J., Messner, B, Turkki, T. and Weissmann, E. (1978). Optical assay of pyruvate carboxylase in crude liver homogenates. *Anal. Biochem.* **86:** 154-158.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **72:**248-558.

Chomcynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate phenol-chloroform extraction. *Anal. Biochem.* **162:** 156-159.

Dannenberg, A.J., Yang, E.K., and Aharon, D. (1983). Dietary lipids induce phase 2 enzymes in rat's small intestine. *Biochem. Biophys. Acta.* **1210:** 8-12.

Demant, P. and Hart, A. (1986). Recombinant congenic strains: a new tool for analyzing genetic traits determined by more than one gene. I*mmunogenetics.* **24:** 416-422.

Dewille, J.W., and Farmer, S.J. (1993). Linoleic acid controls neonatal tissue specific stearoyl-CoA desaturase mRNA levels. *Biochem, Biophys. Acta.* **1170:** 291-295.

Gonzalez, F.J., Tukey, R.H., and Nebert, D.W. (1984). Structural gene products of the Ah locus. Transcriptional regulation of cytochrome P1450 and P3450 mRNA levels by 3-methylchloanthrene. *Mol. Pharmacol.* **26:** 117-121.

Hames, B.D. and Rickwood, D. (1990). One dimensional polyacrylamide gel electrophoresis. In Gel Electrophoresis of Proteins: A Practical Approach, 2 nd ed., Hames, B.D., and Richwood, D., eds., pp. 6-50, Oxford Press, New York.

Isarel, D.I. and Whitlock, Jr. J.P., 1983. Induction of mRNA specific for cytochrome P_1-450 in wild type and variant mouse hepatoma cells. *J. Bio. Chem.* **258:** 110390-10394.

Isarel, D.I., and Whitlock, Jr. J.P., 1984. Regulation of cytochrome P_1-450 gene transcription by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in wild type and variant mouse hepatoma cells. *J. Biol. Chem.* **259:** 5400-5402.

Jones, K.G., and Sweeney, G.D. (1980). Dependence of the porpyrogenic effect of . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin upon inheritance of arylhydrocarbon hydroxylase responsiveness. *Toxicol. Appl. Pharmacol.* **87:** 42-49.

Knutson, J.C., and Poland, A. (1982). Response of murine epidermis to . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: interaction of Ah and hr loci. *Cell.* **30:** 225-234.

Kumaki, K., Jensen, N.M., Shire, J.G.M., and Nebert, D.W. (1977). Genetic differences in induction of cytosol reduced-NADPH: menadione oxidoreductase and microsomal arylhydrocarbon hydroxylase in the mouse. *J. Biol. Chem.* **252:** 157-165.

Martin, B., Marchaland, C., Phillips, J., Chapouthier, G., Spach, C., and Motta, R., 1992. Recombinant congenic strains of mice from B10.D2 and DBA/2: their contribution to behavior genetic research and application to audiogenic seizures. *Behavior Genetics.* **22:** 685-701.

McKim Jr, J.M., Marien, K., Schaup, H.W., and Selivonchick, D.P. (1991). Alteration of hepatic acetyl CoA carboxylase by . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Lipids.* **26:** 521-525.

Mollering, H. (1985). In Methods of Enzymatic Analysis, 3 rd., Bergmeyer. H., and BraB1, M., eds., pp 2-12, VCH, Germany

Nebert, D.W. (1989). The Ah locus: Genetic difference in toxicity cancer, mutation, and birth defects. *Crit. Rev. Toxicol.* **20:** 137-152.

Nebert, D.W., and Gonzalez, F.J. 91987). P450 genes: Structure, evolution and regulation. *Annu. Rev. Biochem.* **56:** 945-993.

Okey, A.G., Vella, L.M. and Harper, P.A. (1989). Detection and characterization of a low affinity form of cytosolic receptor in livers of mice nonresponsive to induction of cytochrome P450 by 3-methylchloanthrene. *Mol. Pharmacol.* **35:** 823-830.

Owens, I.S. (1977). Genetic regulation of UDP-glucuronosyltransferase induction by polycyclic aromatic compounds in mice. Co-segregation with arylhydrocarbon(benzo{a}pyrene) hydroxylase induction. *J. Biol. Chem.* **252:** 2287-2833.

Pace, W. R., Olsen, G. J., and Woses, C. R. (1988). Ribosomal RNA phylogeny and the primary line of evolutionary descent. *Cell.* **45:** 352-326.

Poland, A. , Teitelbaum, P., Glover, E., and Kende, A. (1989). Stimulation of in vivo hepatic uptake and in vitro hepatic binding of [125I]2-Iodo-3,7,8-trichlorodibenzo-p-dioxin by the administration of agonists for the Ah receptor. *Mol. Pharmacol.* **36:** 121-127.

Poland, A. and Glover, E. (1980). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Segregation of toxicity with the Ah locus. *Mol. Pharmacol.* **17:** 86-94.

Poland, A. and Knutson, J.C. (1982). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanisms of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22:** 517-514.

Poland, A.P. and Glover, E. (1976). Stereospecific, high affinity binding of . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. *J. Biol. Chem.* **251:** 4936-4946.

Poland, A.P., Glover, E., Robinson, J.R., and Nebert, D.W. (1974). Genetic expression of arylhydrocarbon hydroxylase activity. Induction of monooxygenase activities and cytochrome P450 formation by . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mice genetically "nonresponsive" to other aromatic hydrocarbons. *J. Biol. Chem.* **249:** 5599-5606.

Sgaramella, V. and Khorana, H. G. (1972). Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymatic joining of the chemically synthesized polynucleotide to form the DNA duplex representing nucleotide sequence 1 to 20. *J. Mol. Biol.* **72:** 427-444.

Silkworth, J.B., Cutler, D.S. and Sack, G. 91989). Teratology of . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a complex environmental mixture from the Love Canl. *Fundam. Appl. Toxicol.* **13:** 1-15.

Sparrow, B.R., Thompson, C.S., Ryu, B.-W, Selivonchick, D.P. and Schaup, H.W., 1994. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced alterations of pyruvate carboxylase levels and lactate dehydrogenase isozyme shifts in C57BL/6J male mice. *J. Biochem. Toxicol.* **9:** 329-335.

Stahl, B.U., Beer, D.G., Weber, L.W.D., and Rozman, K. (1993). Reduction of hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is due to decreased mRNA levels. *Toxicology.* **79:** 81-95.

Weber, L.W.D., Lebofsky, M., Greim, H., and Rozman, K. (1991). Key enzymes of gluconeogenesis are dose-dependently reduced in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Arch. Toxicol.* **65:** 119-123.

Weber, L.W.D., Lebofsky, M., Stahl, B.U., Gorski, J.R., Muzi, G., and Rozman, K. (1991). Reduced activities of key enzymes of gluconeogenesis as possible cause of acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats. *Toxicology.* **66:** 133-144.

Weber, L.W.D., Lebofsky, M., Stahl, B.U., Kettrup, A., and Rozman, K. (1992). Comparative toxicity of four chlorinated dibenzo-*p*-dioxins (CDDs) and their mixture. Part II: Structure-activity relationship with inhibition of hepatic phosphoenolpyruvate carboxykinase, pyruvate carboxylase, and r-glutamyl transpeptidase activities. *Arch. Toxicol.* **66:** 478-483.

Weiberg, M.B., and Utter, M.F. (1979). Effect of thyroid hormone on the turnover of rat live pyruvate carboxylase and pyruvate dehydrogenase. *J. Biol. Chem.* **254:** 9492-9499.

Whitlock, Jr, J.P., (1990). Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* **30:** 251-277.

Whitlock, Jr, J.P., (1993). Mechanistic aspects of dioxin. *Chem. Res. Toxicol.* **6:** 754-763.

Yang, E.K., Radominska, A, Winder, B.S.,and Dannenberg, A.J. (1993). Dietary lipids coinduce xenobiotic metabolizing enzymes in rat liver. *Biochem. Biophys. Acta.* **1168:** 52-58.

Zhang, J., Xia, W. -L., Brew, K., and Ahmad, F. (1993). Adipose pyruvate carboxylase; Amino acid sequence and domain structure deduced cDNA sequencing. *Proc. Natl. Acad. Sci. USA.* **90:** 1766-1770.

Chapter 3
Molecular Cloning of Pyruvate Carboxylase Gene Regulatory Regions and Functional Analysis in Response to TCDD Exposure

Byung-Woo Ryu and Henry W. Schaup

Formatted for submission

3.1 Abstract

The activity and levels of hepatic pyruvate carboxylase (PC), one of the rate controlling enzymes of gluconeogenesis is suppressed by the halogenated aromatic hydrocarbon environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J male mice (Sparrow *et al.* 1994). Suppression is effected at the level of transcription (Ilian *et al.* 1996). Aryl hydrocarbon receptor (AhR) involvement in mediation of the PC suppression was illustrated in previous studies using congenic C57BL/6J $Ah^{b/b}$ mice that have a low affinity TCDD binding receptor (Ryu *et al.* 1995). To better understand the molecular nature of the TCDD-induced suppression of hepatic PC expression, we cloned the PC gene regulatory regions from the congenic $Ah^{d/d}$ mice (upstream and intron 1) using a polymerase chain reaction (PCR) based cloning methods. Cloned constructs were used to study the effect of TCDD in regulation of the PC gene expression using a transient transfection experimental model. The construct reporter was a luciferase gene and the cells were Hepa1c1c7. We report here the DNA sequence of the PC gene regulatory regions and initial investigations delineating their functional role in expression. The PC gene has a TATA-less but completely functional promoter and several *cis*-acting elements which interact with ubiquitous transcription factors that regulate expression of genes coding glycolytic and gluconeogenic enzymes. Consensus sites in the upstream PC gene zone include a CCAAT-box factor, Sp1, Oct-1, CREB, and LF-A1. Of particular interest are, Multiple E-boxes (17 repeats) and a pyrimidine-rich region which forms a triple helical DNA structure. A GAGA factor binding motif is located in the upstream portion of intron 1. The activity of the PC gene promoter is as strong as that of dihydrofolate reductase gene promoter in Hepa1c1c7 cells. This

promoter activity is suppressed by overexpression of human Arnt protein by 70% in contrast to the dose-dependent induction effect of the promoter activity by TCDD. An attempt to assign a functional role to the polypyrimidine regions located in intron 1 was inconclusive when transient transfection assay in Hepa1c1c7 cell was done.

3.2 Introduction

Halogenated dibenzodioxins have generated intensive concern because of their potential toxicity and ubiquitous presence as environmental contaminants. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), one of the most toxic environmental contaminants, has been extensively employed to study dioxin toxicity in animals and humans. It is generally accepted that TCDD toxicity is mediated by intracellular proteins called the Ah receptor and Arnt (Scheuplein *et al* 1991). The Ah receptor and Arnt belong to the basic helix-loop-helix (bHLH) class of transcription factors (Burbach *et al.* 1992; Ema *et al.* 1992). Additionally, these two proteins share a second region of homology, the PAS domain, which is found in the *Drosophila* midline developmental protein Sim (Nambu *et al.* 1991) and the circadian regulator Per (Takahashi 1992). The bHLH domain confers DNA binding properties as well as the capacity to form dimers. The PAS domain is involved in ligand binding and hsp90 binding (Whitelaw *et al.* 1994). The presence of these two domains confers or unique feature to Ah receptor where conferred to the bHLH class DNA transcription factors. Although the TCDD induced signal transduction pathway is incompletely resolved downstream of ligand binding to the Ah receptor, It is generally considered that the sequential events are: a) the dissociation of hsp90 from the Ah receptor, b) nuclear translocation, c) heterodimerization with Arnt, and d) sequence

specific DNA binding (Perdew 1988; Hoffman *et al.* 1991; Hujisawa-Sehara *et al.* 1988; Denison 1988). It is believed that binding of the Ah receptor:Arnt complex to their responsive element enhancer induces the alteration of local DNA chromatin structure, thereby allowing increased promoter accessibility and transcriptional activation of TCDD responsive genes (Morgan and Whitlock Jr. 1992).

One of the most apparent acute toxic effects of TCDD is a wasting syndrome (Max and Silbergeld 1987; Peterson *et al.* 1984), The molecular events associated with this wasting syndrome are unknown. However, animals exposed to sublethal dose of TCDD most likely change their body weight set-point. This is controlled by hormonal regulation (Peterson *et al.* 1984). There are several reports that TCDD affects intermediary metabolism related to glucose. The alteration of $^{14}CO_2$ exhalation rate in ^{14}C-glucose treated rats is detected one day after TCDD exposure (Weber *et al.* 1987). The key enzymes of gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G-6-Pase), and pyruvate carboxylase (PC) are dose-dependently reduced in TCDD treated rats (Weber *et al.* 1991). The reduction of hepatic PEPCK in TCDD treated rats is due to decreased mRNA levels (Stahl *et al.* 1993). In previous reports, we have showed that the activity and levels of PC, one of the rate controlling enzymes of gluconeogenesis, are reduced in C57BL/6J male mice exposed to sublethal dose of TCDD (Sparrow *et al.* 1994). Ilian and his colleagues reported that reduction of PC levels is due to decreased PC mRNA (Ilian *et al.* 1996). Furthermore, Ah receptor involvement in mediation of PC suppression by TCDD was demonstrated by Ryu and his colleagues in study using congenic C57BL/6J $Ah^{d/d}$ mice that produce a low affinity TCDD binding Ah receptor (Ryu *et al.* 1995).

Here, I cloned the PC gene regulatory regions (upstream and intron1) in C57BL/6J mice using an inverse polymerase chain reaction (PCR). This was done to gain insight into the regulatory mechanisms associated with expression in response to TCDD exposure. Primary structural analysis of the PC gene upstream region reveals that the gene has no TATA binding motif (Figure 3.2). This is consistent with other observations which indicate that TATA-less housekeeping genes are constitutively expressed. However, we found many *cis*-acting elements which interact with ubiquitous factors that regulate expression of genes coding for glycolytic and gluconeogenic enzymes (Lamaigre and Rousseau 1994). Examples include CCAAT-box factor, Sp1, Oct-1, cAMP responsive element binding factor (CREB), HNF-4 (LF-A1) (Figure 3.2). In particular, multiple E-box sequences (CANNTG), which is very similar to glucose response element (GIRE) and essential core motif CACGTG of carbohydrate response element (ChoRE, CACGTGNNNGCC) of rat S_{14} gene (Shih and Towle 1992) are found in the upstream region of the PC gene. The first GIRE was found in the promoter of the L-pyruvate kinase gene using transient expression assays in hepatocytes in primary culture (Bergot *et al.* 1992; Liu *et al.* 1993) and in transgenic mice (Cuif *et al.* 1993). The L-pyruvate kinase GIRE nucleotide sequence is CACGGG. This differs from the c-Myc family E-box (CACGTG) by a single nucleotide. Since the PC gene upstream region (1634 bp) contains 17 repeats of the E-box (CANNTG) motifs, we have speculated that these *cis*-acting elements may play pivotal role in regulation of the PC gene expression. Additionally, it has been reported that Arnt protein homodimer which has crucial role in TCDD induced signal transduction in concert with AhR recognizes E-box motifs, CACGTG and CAGCTG (Antonsson *et al.* 1995; Dang et al. 1992). Therefore, it is conceivable that the Arnt

homodimer may affect the regulation of the PC gene. To investigate the functional role of the PC gene upstream regulatory region in connection with Arnt and TCDD, we have done protein transient expression experiments using a Hepa1c1c7 cell culture system. Our findings reveal the PC gene upstream region has strong promoter activity. An analyses of the sequence for DREs in the PC upstream zones reveals that there are no AhR/Arnt heterodimer binding motif within investigated range (1634 bp). Despite the absence of DREs, TCDD increases promoter activity in dose dependent manner, this suggest implicating the existence of non-classical AhR mediated signal transduction pathway by which TCDD may affect the PC gene transcription. We also found that Arnt suppresses the promoter activity of the PC gene upstream region in the presence of TCDD.

In many genes, introns have a functional role in regulation of gene expression by enhancing and/or suppressing promoter activity. They also play a role in mRNA stabilization. Partial sequence data of the Intron 1 of the PC gene reveals that there are potentially unique *cis*-acting elements with which GAGA-factor interacts (Biggin and Tjian 1988; Qin Lu *et al.* 1993). It has been reported that this kind of novel DNA sequence, 21 repeats of TC nucleotides (TC_{21}•AG_{21}) have tendency to form non-B form DNA structures (triple helix) and such zones should be sensitive to S1 nuclease digestion (Panyutin and Wells 1991). To see If there is any significant functional role of the triple helix forming sequences, this region of intron 1 was also investigated using transient expression assay system.

3.3 Experimental Procedures

3.3.1 Materials

Long template DNA polymerase kit were purchased from Bohringher Manhiem (Indianapolis, IN), Cloning vector pBS+ and luciferase reporter vector pGL3-basic were obtained from Stratagene (La Jolla, CA), and Promega (Madison, WI) respectively. Human Arnt expression vector was a generous gift from Dr. Hankinson (UCLA, CA) TCDD was purchased from Midwest Research Institute (Kansas City, MO). The purity (>99%) and concentration of TCDD were confirmed by GC-Mass in Oregon State University (Corvallis, OR). Plasmid DNA purification kits were purchased from QIAGEN (Chatsworth, CA). Other molecular biological reagents including restriction endonucleases, T4 ligase, polynucleotide kinase, and S1 nuclease were obtained from New England Biolabs (Beverly, MA), Life Technologies (Gaithersburg, MD), Sigma (St.Louis, MO), CLONTECH (Polo Alto, CA).

3.3.2 Cloning of PC Gene Regulatory Regions

For PCR cloning of the PC gene regulatory regions, genomic DNA was prepared from C57BL/6J male mice hepatic tissue using Puregene DNA isolation kits from Gentra Systems, Inc. (Minneapolis, MN). This genomic DNA was used in PCR amplifications to obtain intron 1 and the 5'-upstream regions of the PC gene.

To clone intron 1 of the PC gene, two primers were designed, based upon the PC cDNA sequence cloned from mouse adipose tissue (Zhang *et al.* 1993). The primers were synthesized by the Center for Gene Research and Biotechnology of Oregon State University (Corvallis, OR). Forward primer A is 5' GCC TCG GGT GGA GCA GTT ACT 3' and the backward

primer B is 5' GGT GAG ACG TGA GCG AAG TTG 3'. The forward primer A is identical to +18 ~ +38 nucleotides of the leader sequence of the PC gene. The backward primer B anneals to +976 ~ +956 region of PC the coding zone (for the position of primer A and B, see mouse adipose PC cDNA sequence from Zhang *et al* 1993). The reaction mixtures were prepared as described in instruction manual for the Expand^{TM} Long Template PCR System from Boehringer Mannheim including 1mg of genomic DNA from mouse as template. The PCR cycling conditions were template denaturation at 94 ^{o}C for 15 seconds, annealing at 62 ^{o}C for 35 seconds, elongation at 68 ^{o}C for 4 minuted for up to 10 cycles. This was followed by addition of 20 seconds to previous cycle elongation time and cycling continue until the cycling number reached 30. PCR amplification was conducted using Perkin Elmer GenAmp 430 thermal cycler. The product, about 9.1 kb fragment, was identified by electrophoresis through a 1% (w/v) agarose gel in 0.5 X TBE buffer. For convenience, we cloned into the BamH I site of cloning vector pBS^{+} and used an inverse polymerase chain reaction (IPCR) to clone 5' flanking regions. The 9.1 kb fragment was digested by BamH I producing two fragments, 4.1 kb and 5.0 kb fragments. These two BamH I restricted fragments were cloned into a BamH I restriction site of cloning vector, pBS^{+} phagemid, as described elsewhere. Transformation was accomplished by heat and $CaCl_2$ shock of XL1-Blue MRF^{-} competent cells, obtained from Stratagene (La Jolla, CA), and colony identification was done via b-galactosidase/IPTG blue/white screening (Sambrook *et al.* 1989). Two clones named pBS^{+}/pci4.1 and pBS^{+}/pci5.0 respectively were done. Restriction mapping on the cloned DNA revealed that pBS^{+}/pci4.1 has an insert harboring the 5'end to BamH I site of Intron I of the PC gene (Figure 3.1). Sequencing was done from both ends of the inserts using a

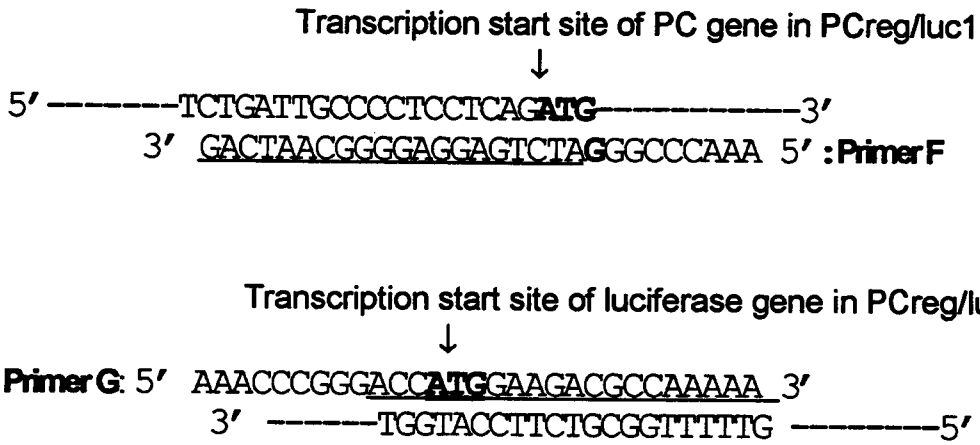
Applied Biosystems model 377 and 373A automated DNA sequences (Figure 3.6) in the Center for Gene Research and Biotechnology at Oregon State University (Corvallis, OR). To confirm that we had the correct DNA fragment for intron 1, a second PCR was conducted using primer A and another backward internal primer C; 5' CTC CAG ACG CCG GAC ATT TGG 3' located at +197 to ~ +167 (for the position of primer C, see mouse adipose PC cDNA sequence from Zhang *et al* 1993) of the PC coding sequence using the same PCR conditions described above. The amplified product was directly sequenced using primer C as a sequencing primer. The 5' and 3' splice junctions of intron 1 were identified by direct sequence comparison of these sequence data with the adipose PC cDNA (Zhang *et al.* 1993), (Figure 3.6).

To isolate upstream regions of the PC gene, inverse polymerase chain reaction (IPCR) were employed (Ochman *et al.* 1990). To prepare endonuclease restricted templates for IPCR, restriction enzymes Avr II, Sph I, Pvu II, EcoR I, Hind III, Xba I. Taq I, and Hae III were selected. Two micrograms of mouse genomic DNA was restricted with each of the enzymes. These digested genomic DNA templates were purified by phenol and phenol:chloroform:isoamylalchol(25:24:1) extraction followed by 95% EtOH/0.8 M LiCl precipitation. The samples were rehydrated with TE buffer (pH 8.0), and ligated with T4 DNA ligase at 14 °C for 16 hours. This generates circular double strand DNA . These circular DNA templates were linearized by cutting with BamH I endonuclease. After EtOH precipitation, these templates were redissolved and used for IPCR. To amplify unknown 5' flanking regions by IPCR, It is necessary to have reversely oriented two primers which flank one restriction enzyme site in the known region of the gene. These two primers are designed using exon 1 of the PC gene,

Primer D: 5' GGC AGT AAC TGC TCC ACC CGA G 3' and primer E: 5' CTA TCA CGT CCT CTG TG TGC 3' which annealed to +66 ~ +45 and +77 ~ +96 region respectively and also flank the BamH I site located in exon 1 of the PC gene (Figure 3.2). The reaction and cycling condition were as described before. The reaction using templates digested by Pvu II and Hind III were generated 1.2 kb and 5.0 kb DNA fragments named IPCR1.2 and IPCR5.0 respectively. After purification by phenol:chloroform:isoamylalcohol extraction and EtOH/LiCl precipitation, IPCR1.2 was incubated with the *E. coli* klenow fragment to create blunt ends and cloned into a Sma I site of pBS$^+$ phagemid generating pBS$^+$/IPCR1.2. After purification by phenol:chloroform:isoamylalcohol extraction and EtOH precipitation, IPCR5.0 was digested with Hind III producing two fragments sized 1.75 kb and 3.25 kb as expected. The name of IPCR1.75 was designated for the 1.75 kb small fragment which constitutes the 5' flanking region of the PC gene. This IPCR1.75 was purified by 1% (w/v) low-temperature melting agarose gel followed by b-agarase digestion (New England Biolabs, Beverly, MA) and isopropanol precipitation. This purified IPCR1.75 was inserted into a Sma I and Hind III digested site of pBS$^+$ phagemid generating pBS$^+$/IPCR1.75 vector. Sequencing was done twice from both ends of inserts by sequencing sense and antisense strands. These sequence data were analyzed using the software, MacVector and AssemlyLine. To search for transcription factor binding consensus cis-acting elements the software Signal Scan(version 4.05) supported by Bioinformatics and Molecular Analysis Section (BIMAS) was employed.

3.3.3 Construction of Luciferase Expression Vectors

To generate the luciferase expression vector PCreg/luc2, which has the upstream and intron 1 of the PC gene, we used a pGL3-basic reporter vector harboring the luciferase gene (Promega). The insert consist of a 1.4 kb upstream sequence plus exon 1 and intron 1 of PC gene. This was generated using Expand^{TM} Long Template PCR Kit and using foreword primer F: 5' AAC ACC TTC CCA AGA GGC AC 3' located in upstream region (-1359 ~ -1340) (Figure 3.2). The backward priming was done with primer C, mouse genomic DNA (1 μg) served as a template. The reaction and cycling conditions were the same as described for cloning intron 1. Amplified product of about 8.6 kb was purified by polyethylene glycol (PEG) as described in Current Protocols in Molecular Biology, Volume 2 (Ausubel *et al.* 1987) and directly sequenced using sequencing primer G: 5' GCA TGC TAG CTT TCA ATT GGG 3' which corresponds to positions of 75 to ~ 95 of intron 1 (Figure 3.6). This was done to confirm that the product originated from the PC gene regulatory regions. Following agarose gel purification and blunt ends were created using the *E. coli* Klenow fragment. The product was inserted into a Sma I site of the pGL3-basic luciferase vector producing a 13.4 Kb reporter vector designated PCreg/luc1. Since this vector has intact intron I and part of exon 2, which contains transcription start site of the PC gene, it was necessary to mutate the PC gene transcript start site in order to maintain the proper open reading frame for the firefly luciferase gene. It was also essential to maintain intron splice junction sequences. Therefore, we designed and synthesized two primers reversely oriented to each other to amplify by PCR a subclone of PCreg/luc2 with the correct open reading frame for luciferase gene from PCreg/luc1 as shown below.



The reaction mixture composition was prepared by following protocols as discribed in instruction manual of Expand™ Long Template PCR kits including 10 ng PCreg/luc1 as a template were used in these amplification. The PCR cycling conditions were denaturation at 94 °C for 15 second, annealing at 61 °C for 35 second, elongation at 68 °C for 8 minutes 20 seconds up to 11 cycles and then 20 seconds were added to previous cycle elongation times. The cycling was continued to 31. The PCR amplified product was digested by Sma I after removal of unbound excess primer by filtration through Microcon 50 (Amicon, Inc., Beverly, MA). The material was ligated using T4 DNA ligase for 16 hours at 14 °C. Competent cells, XL1-Blue MRF-, were transformed with the ligated PCR product by $CaCl_2$ and heat shock treatment. The PCreg/luc2 was isolated and purified from the transformed cell colonies using Qiagene plasmid isolation kits (Chatsworth, CA). The open reading frame of the luciferase gene in PCreg/luc2 reporter vector which is driven by PC gene promoter was conformed by dideoxy DNA sequencing.

The triple helix region deletion mutant reporter vector designated PCreg/luc/thM was subcloned from PCreg/luc2 using the PCR based

subcloning strategy described for the construction of PCreg/luc2. Reversely oriented primers were designed using the 5' region of intron 1 DNA sequence. Primer H: 5' CTA GCA ATC CCA ATT GAA AGC 3' and primer I: 5' TAT GTG TGT TGG TGG CTG GT 3' were anneal respectively to the positions 103 to ~ 83 and 251 ~ 270 in intron 1 (Figure 3.6) Another deletion mutant vector, PCreg/luc/C, which has an insert with a deleted 5' end of intron 1 splicing junction was constructed from the Pcreg/luc2. This was accomplished using PCR with two reversely oriented primers designed from position 112 ~ 95 nucleotide position of exon 1; primer J 5' AGC ACC TAG GTG TGC TGC 3' (Figure 3.2) at position 534 to 561 in intron 1 and primer K 5' GGC TTT GAT GGA GGG CCT T 3' (Figure 3.6).

A plasmid PCp/luc which harbors DNA from proteins -750 to +66 of the C57BL/6J mouse PC promoter was constructed for the analysis of promoter activity. The insert was amplified from pBS+/IPCR1.75 vector by PCR using forward primer L: 5' AGA CGG TTC TGT CCC TTC AG 3' which corresponds to -750 ~ -730 region (Figure 3.2) and backward primer D. The reaction mixture was prepared as described previously. Thermal cycling conditions were: pre-amplification denaturation (1 cycle): 94 °C for 30 seconds, thermal cycling (32 cycles): denaturation; 94 °C for 30 seconds, annealing; 61 °C for 30 seconds, extension; 68 °C for 1.5 minutes. The 816 bp insert was treated with DNA polymerase I large fragment to create 5' and 3' blunt ends and ligated into Sma I site of pGL3-basic resulting in the generation of PC a promoter directed luciferase expression vector PCp/luc.

As a positive control reporter vector, pDHFR/luc, in which luciferase gene transcription is driven by murine dihydrofolate reductase (DHFR) promoter was constructed. A plasmid pWTluc containing sequences -270 to +20 of the murine DHFR promoter was obtained from Dr. Peggy J.

Farnham (University of Wisconsin, Madison, WI). The Hind III digestion product of pWTluc was isolated by 1.5% (w/v) low-temperature melting agarose gel and inserted into pGL3-basic luciferase vector at the Hind III site located in +30 in the luciferase cDNA to create pDHFR/luc.

For the human Arnt overexpression experiments, a plasmid, pBM5/NEO/M1-1, containing human Arnt cDNA was obtained from Dr. Oliver Hankinson (UCLA. Los Angeles, CA).

3.3.4 Cell Cultures, Transient Transfection, Chemical Treatment

Wild type (wt) mouse hepatoma (Hepa1c1c7) cells derived from a C57BL mouse liver tumor were obtained from J. P. Whitlock, Jr. (Stanford University). They were maintained as monolayers at 37 ^{o}C and 5% CO_2 in a-minimum essential medium without nucleosides, supplemented with 10% fetal bovine serum (FBS). For the transient transfection, Hepa1c1c7 cells were grown to about the 80% confluence stage. These were transfected with desired plasmids using either CLONfectin (CLONTEC Laboratories, Inc., Palo Alto, CA) or polybrene (Aldrich Chemical Co., Milwaukee, Wis.) followed by dimethyl sulfoxide (DMSO) treatment. In the transient transfection experiment using the CLONfectin, 4 x 10^5 cells were plated into 35 x 15 mm cell culture dishes and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. Freshly prepared liposome solution consisted of 4 µg plasmid DNA, 4 µg CLONfectin and serum-free minimum essential medium as described in the CLONfectin^{TM} User Manual. The transfecting solution was gently applied to the cells after removal of the medium from the culture to be transfected. The cells were incubated at 37 °C for 4 hours in a CO_2 incubator. After removal of the CLONfectin/DNA-containing medium, the cells were washed with 1X Dulbecco's phosphate buffered

saline (PBS). They were grown in 2 ml 10% FBS containing minimal essential medium for 24 hours at 37 °C in a 5% CO_2 incubator. For the transient transfection using polybrene, 4 x 10^5 cells seeded into 60 x 15 mm cell culture dishes and grown at 37 °C for 24 hours. They were then treated with 2 ml of absorption cocktail containing 10 ~ 30 µg plasmid DNA and 30 µg polybrene for 6 hours with occasional shaking to enhance adsorption as described (Kawai and Nishizawa 1984). The culture solution was then removed, and the cells were treated with 3 ml medium containing 30% DMSO (Sigma Chemical Co., St. Louis, MO) at 37 °C for 4.5 minutes. The culture was washed twice with 4 ml complete medium and incubated with 4 ml fresh medium at 37 °C for 48 hours. For the TCDD treatment, 8 X 10^5 cells were plated in 75 cm^2 cell culture flask (Corning) and transiently transfected with 8 µg DNA plasmid using CLONfectin as described above. The cells were trypsinized with 1 ml trypsin-EDTA (0.05%-0.02%) to detach the cells from the flask, then collected, mixed by brief vortexing, divided by two aliquots. Cells were then plated in 35 x 10 mm dishes containing 2 ml complete medium with and without TCDD various doses dissolved in 4 ml of DMSO. The cells are grown at 37 °C in a 5% CO_2 incubator for 24 hours. The cells grown in medium without TCDD were used for control treatments.

3.3.5 Luciferase Activity Assay

The tissue culture dish was transferred onto ice, the media were removed, and then the plates were rinsed twice with cold 1X PBS. Cells were lysed at room temperature for 15 minutes with 100 ml and 200 ml of luciferase lysis buffer (Promega) for 30 mm and 60 mm dishes respectively. The lysed cell sample was scraped manually, transferred into a clean microfuge tube, and rapidly frozen (-85 °C) then thawed at room

temperature. Lysed cells were vertexed and cell debris was pelleted by centrifugation at 12,000 x g in a microcentrifuge for 15 seconds at room temperature. The supernatant was transferred to a fresh tube. Luciferase activity in an aliquot of cleared lysed cell supernatant (20 ml or 40 ml) was determined by mixing it with 100 ml of luciferase assay reagent (Promega) and measuring the resulting luminescence in a LKB Luminometer 1250. Luciferase activity was standardized to the protein concentration of the lysed cell supernatant. Protein concentration was determined using Bradford protein assay (Bradford 1976) with bovine serum albumin serving as the protein standard. Activity was expressed in relative luminescence units (RLU) per microgram of protein.

3.3.6 S1 Nuclease Assay

For the S1 nuclease digestion assay, a plasmid, pBS$^+$/pci/th1.0, containing the triple helix forming DNA sequence originating from the 5' region of intron 1 of the PC gene (Figure 3.1) was subcloned from pBS+/pci4.1 vector. An Avr II digested 1.0 Kb fragment from pBS+/pci4.1 was isolated and purified electrophorectically from a 1% (w/v) low-temperature melting agarose gel treated with b-agarase followed by isopropanol/LiCl precipitation. Both ends of the fragment were modified by *E. coli.* DNA polymerase I large fragment (Klenow) to create blunt ends and then ligated into Sma I site of pBS$^+$ cloning vector resulting the pBS$^+$/pci/th1.0 vector. Orientation of the insert was determined by restriction analysis.

S1 nuclease from *Aspergillus oryzae* was obtained from Life Technologies. Reaction conditions for S1 nuclease were modified from those described by Singleton *et al.* (Singleton *et al.* 1982). For the S1

nuclease digestion, pre-incubation was carried out in a total volume of 300 ml at a DNA concentration of 3.3 µg/ml for 30 minutes at 37 °C. The reaction buffer consisted of 30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM zinc sulfate, and 5% (v/v) glycerol. S1 nuclease (5 Units) was added and then the reaction mixture was incubated further at 37 °C for 40 minutes. Reactions were stopped by placing the mixture on ice and adding EDTA to a final concentration of 30 mM. The S1 nuclease digested DNA was then purified and isolated by phenol:chloroform:isoamylalcohol extraction twice, precipitation with 7.5 M ammonium acetate (0.5 volume) and 100% EtOH. The position of the S1 nuclease cut(s) was mapped by Hind III and Nde I restriction followed by 1.5% (w/v) agarose gel electrophoresis at 40 V constant voltage for 4 hours .

3.4 Results

Regulatory regions of the PC gene (Figure 3.1) were cloned using PCR based cloning methods in order to characterize their functional role in transcriptional regulation of the PC gene. To clone the upstream region of the PC gene (1634 bp) using IPCR method, restriction mapping of intron 1 was of pre-requite. This was required to enable select on restriction of endonucleases which could be used for the preparation of templates for IPCR. Therefore, intron 1 of the PC gene was cloned as described in experimental procedures and restriction mapping was done on the 5' side of BamH I digestion fragment (4.0 Kb) of the intron 1. The PC gene is 7.0 kb in length of intron 1. Within the first 200 bp there are pyrimidine rich sequences which may posses a novel DNA structure including triple helix and GAGA-factor, binding site (Biggin and Tjian 1988; Qin Lu *et al.* 1993). Two different l5' upstream fragments (1634bp and 210bp) of the PC gene

Figure 3.1 Regulatory zone for C57BL/6J male mouse liver pyruvate carboxylase gene. A fragment of intron 1 (7.0 kb) was amplified using PCR, cloned into pBS+ phagmid. The restriction map was constructed as described in experimental procedures. The first Pvu II and Hind III restriction sites were utilized for Long Template Inverse Polymerase Chain Reaction (IPCR) to amply unknown 5' flanking regions (1643bp). The DNA sequence data (Figure 3.2 and Figure 3.6) reveal that pyruvate carboxylase gene regulatory region has multiple E-box motifs in 5' upstream and what appear to be degenerate DREs bounded by the pyrimidine rich region of intron 1.

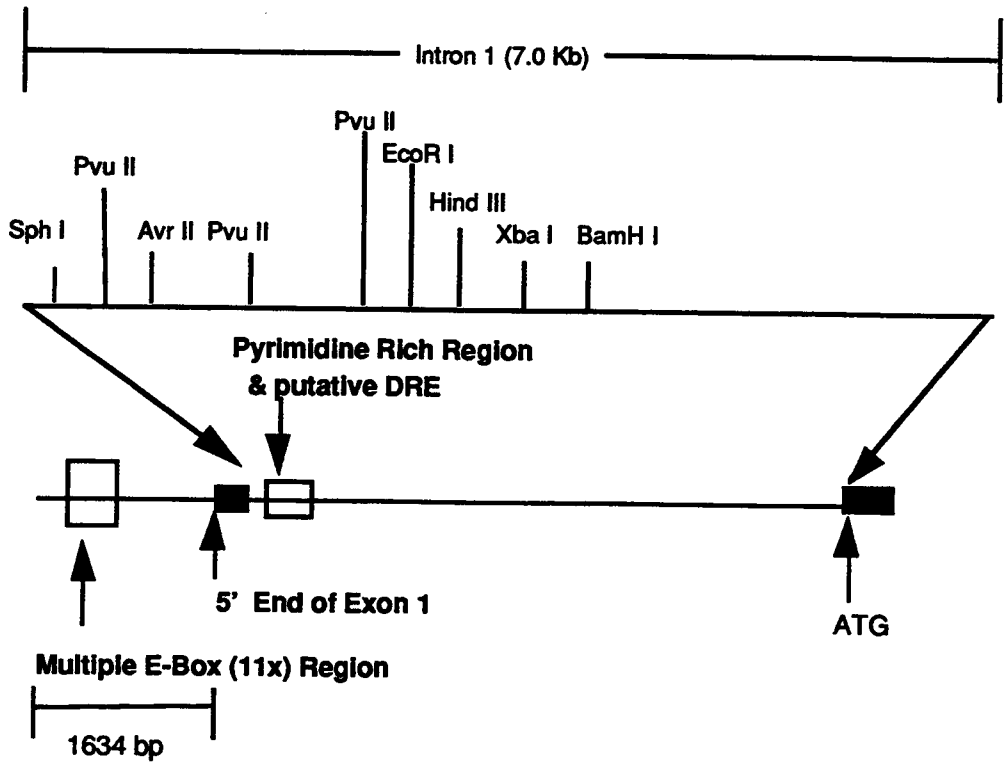

Intron 1 (7.0 Kb)
Pvu II
EcoR I
Pvu II
Hind III
Sph I
Avr II
Pvu II
Xba I
BamH I
Pyrimidine Rich Region
& putative DRE
5' End of Exon 1
ATG
Multiple E-Box (11x) Region
1634 bp

Figure 3.1

were generated using Inverse PCR. Independently prepared genomic DNA templates restricted by Hind III and Pvu II were employed respectively. DNA sequence analysis reveals that the 217bp overlapping region of the two upstream fragments are identical, indicating that those fragments are actually originated from upstream portion of the PC gene. Figure 3.2 shows the DNA sequence to -1634, portion. The PC gene has TATA-less promoter. A pyrimidine rich initiator (Inr, consensus YYANt/aAYY). This represents one of core-promoter elements defined as a minimal DNA element that is necessary and sufficient for accurate transcription initiation by RNA polymerase II (Roeder 1996). It is located near transcription initiation site. The potential PC gene Inr core sequences, +7CTAGTGC+13, differs in only one nucleotide from consensus sequence (YYANt/aYY) and it is located in an acceptable right position. Two GC factor (GCF) binding sites are in further upstream of the Inr located (positions -48 ~ -39 and -94 ~ -85) . Some common *cis*-acting elements found in the upstream region of genes encoding glucose metabolizing enzymes (Lemaigre and Rousseau 1994) are also localized in the PC gene promoter region. These cis-acting elements are Sp1, CCAAT box factors (CTF/NF-1), octamer factor (Oct-1), and c-AMP response element binding protein (CREB/ATF) as indicated in Figure 3.2. The presence of hormone response elements such as the glucocorticoid response element (GRE) as well as cAMP response element (CRE) is expected because PC is one of the enzymes responsive to hormone signals (Lamaigre and Rousseau 1994). One distinctive feature of PC gene upstream region is the presence of numerous E-boxes (17 repeats) within investigated range. Of particular interest are the consecutive 11 repeats of E-boxes located between -1185 ~ -757 wherein 39 nucleotides 5' CTC TCT **CAC CTG** a/gCC CCC AGG CTC TAT GAT ACC CCT

Figure 3.2 DNA sequence of C57BL/6J $Ah^{d/d}$ mouse liver pyruvate carboxylase gene upstream region and non-protein coding exon 1. The PC gene 5' upstream fragment (1634bp) was amplified from C57BL/6J male mouse genomic DNA using Long Template Inverse Polymerase Chain Reaction (LTIPCR) as described in experimental procedures section. The sequencing was done at least in duplicate sequencing sense and antisense strands. Transcription start site was determined by Barney Sparrow *et al.* (1997). Transacting element binding sites are identified and placed below the underlined consensus sequence.

-1634 5'AAGCTTCAGGCTTAATGCAAACTTCAGGTCTCCACTATTGATGTCACCAAGTAGGAGGG
OCT-1
-1575 CTTTCCCTCAGAGGATTTAAATTGGAGTCTGAACCATTGTCAACTACGAGGACTTCATGGT
CTF/NF1
-1514 AACCATCCAACCAAGTACTCCCCTAATAGGGTACTGTGTGGTCTAAGTGAAAGCCTGCCCC
-1453 CCATTCCCACTACTCTCTCTAGCCATGCTATCTTTTGGGATGAGGATGACAGGAGACATCT
E-box
-1392 GGAATAGTGCCAGCCTGAAGGGCTGTAATTGGAAACACCTTCCCAAGAGGCACTGTTGTGA
CTF/NF1
-1331 GGAAAGAGGATGGTCACATTAGATTAGGATGCCAGGACAGGTTGGGTCCCCTTTAGTACCC
-1270 AAACGAGGAATTCTGGGGGGCTTGGAGGGTACCACACTATTCAGTGCCCACCCAAGGTCTT
-1209 CCTACCACCCTAGGTGCTCTGCTTCTCTCTCACCTGACCCCCAGGCTCTATGATACCCCTG
E-box
-1148 AGCTCTCTCACCTGACCCCCAGGCTCTATGATACCCCTGAGCTCTCTCACCTGACCCCCAA
E-box E-box
HNF-4/LF-A1
-1087 GCTCTATGATACCCCTGAGCTCTCTCACCTGGCCCCCAAGCTCTATGATACCCCTGAGCTC
E-box
-1026 TCTCACCTGACCCCCAGGCTCTATGATACCCCTGAGCTCTCTCACCTGACCCCCAGGCTCT
E-box
HNF-4/LF-A1
-965 ATGATACCCCTGAGCTCTCTCACCTGGCCCCCAAGCTCTATGATACCCCTGAGCTCTCTCA
E-box
-904 CCTGACCCCCAGGCTCTATGATACCCCTGAGCTCTCTCACCTGACCCCCAGGCTCTATGAT
E-box E-box
HNF-4/LF-A1
-843 ACCCCTGAGCTCTCTCACCTGACCCCCAGGCTCTATGATACCCCTGAGCTCTCTCACCTGG
E-box
-782 CCCCCAAGCTCTATGATACCCCTGAGCTGTGCAGACGGTTCTGTCCCTTCAGCTTTGAGAA
-721 TTTGCTGAGACTCAGGTGCAACTTGGTCAACCTTATCACTGGAGTTATCTCAGACCCTGGA
E-box
-660 ACTCAACACTCCGTTGGCTGTCACAACATCTGTGGGGCCCTAAGGGGACCCAGTGGGTCCA
E-box
-599 CAGCTGTCCTGCGGCTGGATCTCTCTGGCTGATCATGATTTTAAAAGTTCCTGAGTGTAGG
E-box
-538 CCTGTGAGGGCAGTGGGATTCATTCAGTGAGGGAAAGGCTAGATCAGCTATGGAGATGCTT
CRE
-477 AGGAACAAGGTGGTATGTTCTCTTGTCTGTGACGGCCTTGTCTTGTGTCTGGCAGTGCATT
CRE CRE
-416 GAATTAGGGGAGAGGCTGGTTGCAGCACAGGCCTCCTGTGACTGTAGTAGTGAACCAGCCT
Sp1
-355 TCAGGCTAATGTGACCCTTGCCCTCCATCATGCCTGGCACAGTCCCTACCAAGCAGCCTGT

Figure 3.2

(Continue Figure 3.2)

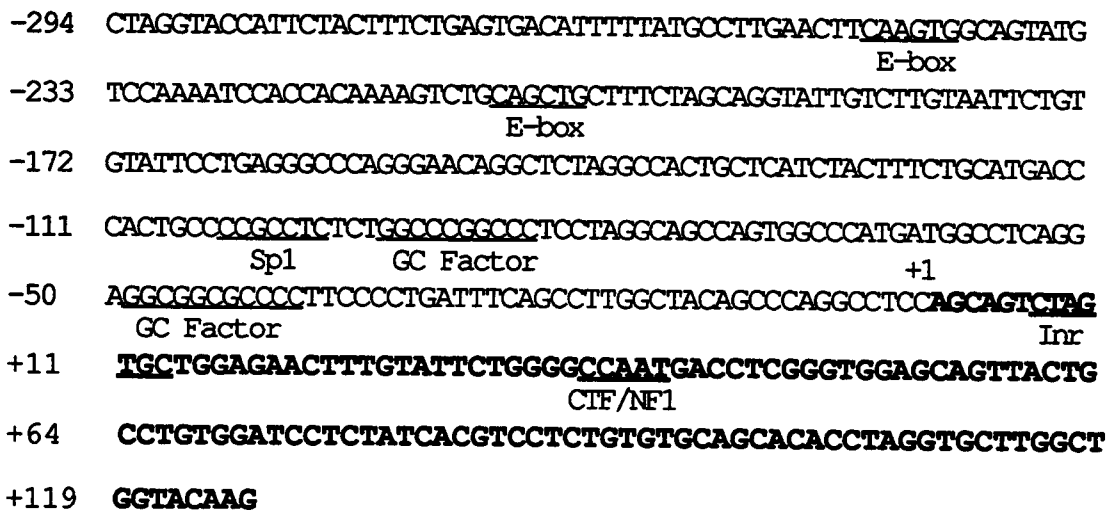


Figure 3.2

GAG 3' containing E-box motif, CACCTG, is repeated 11 times. Three E-box motifs at positions -1062 to -1057, -945 to -940, and -789 to -784, are overlapped with the nucleotide sequence, 5' CTC TCT **CAC CTG** GCC C 3' which may be recognized by liver enriched transcription factor, HNF-4/LF-A1 (consensus, TGGACt/ct/c$(N)_{1-4}$TGGCCC) known as essential for carbohydrate induction in L-pyruvate kinase (Liu *et al.* 1993).

The promoter activity of upstream zones (–750 to +66) PC was determined using luciferase reporter gene (PCp/luc) in Hepa1c1c7 mouse hepatoma cell transient expression assays. Figure 3.3 shows approximately 25-fold induction of promoter activity relative to control vector (pGL3-b) which harbors no promoter. The PC gene upstream zone (from transcription start site to -750) has five of the 17E-box motifs: CAGCTG (-209 to -204, -599 to -594), CAAGTG (-247 to -242), CATCTG (-634 to -629), and CAGGTG (-710 to -704). These E-box motifs are similar to glucose response element (GlRE, CACGGG) in L-pyruvate kinase (Shih and Towle 1992) and carbohydrate element (ChoRE, CACGTGNNNGCC) in rat S_{14} gene (Bergot *et al.* 1992; Liu *et al.* 1993; Cuif *et al.* 1993). It has also been reported that Arnt homodimer recognizes and binds to E-box motifs CACGTG and CAGCTG (antonsson *et al.* 1995). Therefore, we decided to co-transfect the Hepa1c1c7 cell with PCreg/luc and pBM5/NEO/M1-1 a construct that has a constitutively expressed human Arnt cDNA. In these experiments the overexpressed of Arnt suppresses PC promoter activity by 30% relative to the PCp/luc alone (Figure 3.3). This suggest that the E-box motifs found the PC gene promoter may have some functional role involving Arnt. The effect of TCDD on PC promoter activity in Hepa1c1c7 cell was investigated. Figure 3.4 shows that TCDD enhances the promoter activity in dose-dependent manner over the investigated dose range. It should be

Figure 3.3 Promoter activity of the pyruvate carboxylase gene upstream promoter and overexpression effect of human Arnt . Hepa1c1c7 cells (4 x 10^5 cells/35 x 10 mm dish) were transiently transfected with 4 μg of the PC gene promoter (-750 to +66) containing reporter vector (PCP/luc) and 4 μg CLONfectin (CLONTEC, Palo Alto, CA). Luciferase activity was measured as described in experimental procedures. For the Arnt overexpression, 4 μg PCp/luc + 4 μg pBM5/NEO/M1-1 (human Arnt cDNA containing) plasmid and 8 μg of CLONfectin were used for the transient transfection. As a control vector, pGL3-Basic firefly luciferase was used. The luciferase activity for pGL3-Basic vector is standardized to 1, therefore the y axis represents the ratio between PCp/luc and pGL3-Basic. The *bar graph* shows the mean and standard deviation for three independent DNA transfection experiments for Hepa1c1c7.

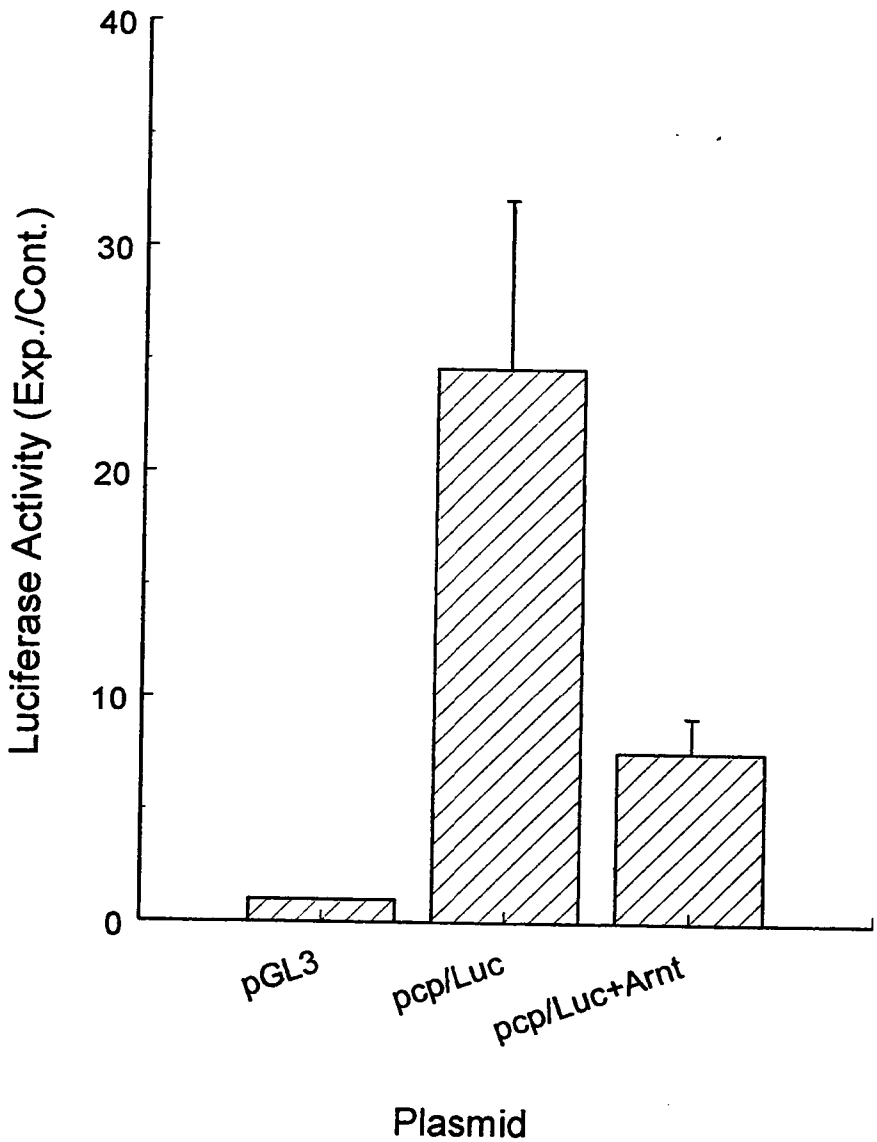

40
30
20
10
0
Luciferase Activity (Exp./Cont.)
pGL3
pcp/Luc
pcp/Luc+Arnt
Plasmid

Figure 3.3

Figure 3.4 Dose-responsive induction effect of TCDD on the promoter activity of PCp/luc in Hepa1c1c7. Hepa1c1c7 cells (8×10^5 cells/75 cm^2 culture flask) were transiently transfected with 8 μg of the PC gene promoter (-750 to +66) containing reporter vector PCp/luc and 8 μg of CLONfectin (CLONTEC, Palo Alto, CA). Transfected cells were divided by two and plated into 35 x 10 mm cell culture dishes as described in experimental procedures. One group of cells are treated with TCDD at concentration of 1 mM to 0.1 nM range. The other group of cells receiving no TCDD served as control. The y axis represents the ratio of luciferase activities between TCDD treatment and control. The *graph* shows the mean and standard deviation for three independent DNA transfection and TCDD treatment experiments for Hepa1c1c7.

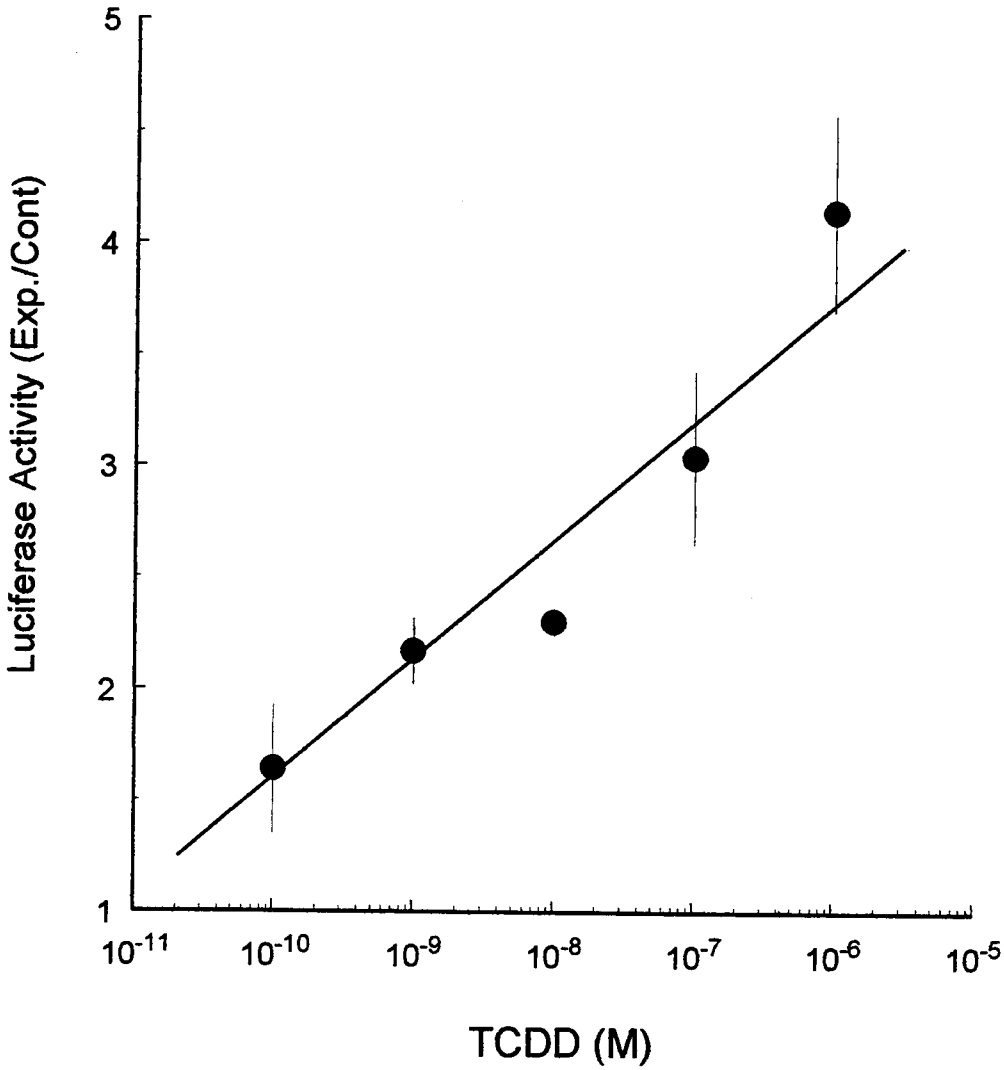

Luciferase Activity (Exp./Cont)
5
4
3
2
1
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
TCDD (M)

Figure 3.4

noted that the PC promoter driving the luciferase reporter vector PCp/luc has no DRE. This result suggest the existence of a non-AhR mediated signal transduction pathway by which TCDD induces increased promoter activity in the transient transfection experiments using Hepa1c1c7 cell. The effect of TCDD on the PC promoter activity of PCp/luc when the Arnt protein is overexpressed in Hepa1c1c7 was also employed. Figure 3.5 shows that co-transfection with the human Arnt gene overrides the effect of a dose-dependent increasing promoter activity by TCDD treatment. However, promoter activity remains 3- to 3.5-fold induction over controls in manner of slight enhancement as the TCDD dose is increased. It is interesting to observe that approximately 3-fold induction of the PC promoter activity with Arnt overexpression relative to about 1.5-fold induction without Arnt co-transfection (Figure 3.4) at the lowest concentration (0.1 nM) of TCDD treatment . The results shown in Figure 3.4 and in the Arnt overexpression experiment (Figure 3.5) imply that TCDD and Arnt may distinctively affect to the PC promoter activity.

Partial DNA sequence analysis of the 5' and 3' end of intron 1 from 5' is shown in Figure 3.6. Two pyrimidine rich regions, located at positions +112 to +153 and +210 to +249 may have non-B form DNA structure. Degenerate DRE core sequences (+174 ACAGCGTG +169,) are located in the region flanked by those two pyrimidine rich sequences.

DNA with homopyrimidine homopurine sequences have a propensity to form triple helices that are known to be hypersensitive to S1 nuclease digestion (Panyutin and Wells 1991). The intermolecular triple helix formation occurs upon binding of the third oligopyrimidine strand in the major groove of double-stranded DNA, in a parallel orientation with respect to the homopurine strand of the double helix (Le Doan *et al.* 1987). These

Figure 3.5 Luciferase induction by TCDD on the promoter activity of PCp/luc in Hepa1c1c7 cells over-expressing human Arnt. Hepa1c1c7 cells (8×10^8 cells/75 cm^2 culture flask) were transiently transfected with 8 μg of the PC gene promoter (-750 to +66) containing reporter vector PCp/luc + 8 μg pBM5/NEO/M1-1 (human Arnt cDNA containing) plasmid and 16 μg of CLONfectin (CLONTEC, Palo Alto, CA). Transfected cells were divided by two and plated into 35 x 10 mm cell culture dishes as described in experimental procedures. One group of cells are treated with TCDD at concentration of 1 mM to 0.1 nM range. The other group of cells receiving no TCDD were served as control. The y axis represents the ratio of luciferase activities between TCDD treatment and control. The *graph* shows the mean and standard deviation for three independent DNA transfection and TCDD treatment experiments for Hepa1c1c7.

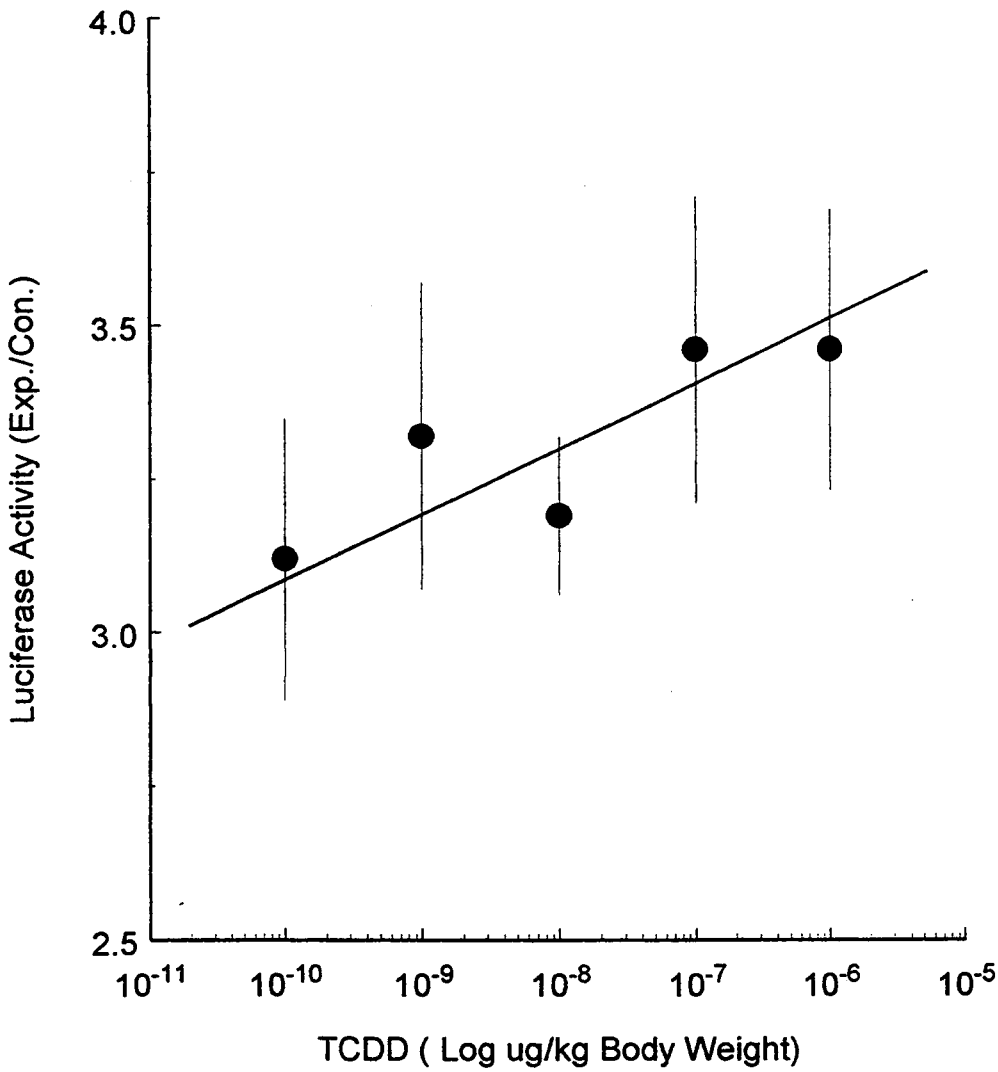

4.0
3.5
3.0
2.5
Luciferase Activity (Exp./Con.)
10-11
10-10
10-9
10-8
10-7
10-6
10-5
TCDD (Log ug/kg Body Weight)

Figure 3.5

Figure 3.6 Partial DNA sequence of C57BL/6J $Ah^{d/d}$ mouse liver pyruvate carboxylase gene intron1. The PC gene intron 1 fragment (7.0 kb) was amplified from C57BL/6J male mouse genomic DNA using Long Template Polymerase Chain Reaction (LTPCR) as described in experimental procedures section. The amplified DNA fragment was sequenced using an automated nucleotide sequencer (Applied Biosystems model 377 and 373A). The sequencing was done in duplicate by sequencing sense and antisense strands. The Pyrimidine rich regions, GAGA factor binding sites, TFII-D binding TATA boxes, putative dioxin response element (DRE) and translation start site coding methionine are indicated. The numbering is from position 1 in the intron which begins a position 127 from the transcription start site.

1 5'GTAATTGTTCCAGAGCAGGTATGGCTCTATAAATCTGAATCAACTGCTAGGCTAGGGTTTT
TFII-D

62 ACATGTGATGAAAGCATGCTAGCTTTCAATTGGGATTGCTAGAGGACTAGTCTCTCTCTCTCT

125 CTCTCTCTCTCTCTCTCTCTCTCTCTCACTCGCTCTGTATGTGTGCGACAGTGTGTGTCTC
Triple helix/GAGAfactors DRE

188 CTTCTCTGTGTGTTTCTGTGTGCCTCTGTCTCTCTGTCTCTGTCTCTTGTCTCTCTCTCTCTG
Triple helix/GAGA factors

251 TGTATGTGTGTTGGTGGCTGGTACAAGGTAATTGTTCCAGAGCAGGTATGGCCCTATAAATCT
TFII-D

314 TAATCAACTGCTAGGCTAGGTTTTTACATGTGATGAAAGCATGCTCTGTGCTAGAGGACTAGT

377 CTGTCTGTCTGTCTGTCTGTCTCTCTCTGTCTGTCTGTCTCTCTCTCTCTCTGTGTCTGTCTC

440 TTTTTGTGTGTATGTGTTGGTATGACTCATATCAATATCCTACTCAGAAAACCTTGGAGGGAT

503 TGGTATATAGCAGACTTCATGCTACCTCTATACAGAGAAAGGCTTTGATGGAGGGCCTTGGGG

566 CAGTTAACAAGAAGACCTTGGTGTATATACATGCATGTGTGAACATGTATGTATGTATATGTA

629 TGTGTATGTGTATGTGTATGTGTATGTGTATGTATATGTATATGTATATGTATATGTATATGT

692 ATATGTATAAGGATGCTGGTGTGATCTGTATA.....\\.....ATGTGGGCTGCTCAGTA

GCCTGCTCTAACCTAGACTCTGATTGCCCCTCCTCAG**ATGCTGAAGTTCCAAACAGTTCGAG**

Figure 3.6

novel DNA structures are found in the regulatory regions of numerous highly active genes (Evans and Efstratiadis 1986). S1 nuclease is specific for single strand DNA . One of the characteristics of triple helix conformational structure is the presence of single strand DNA that is displaced with formation of triple helical structure and is a target strand attacked by S1 nuclease. If the homopyrimidine sequence, TC_{21}•AG_{21}, found in the intron 1 of the PC gene has triple helical structure, the plasmid, pBS+/pci/th1.0, should be S1 nuclease sensitive in the position of the triple helices(Figure 3.7). The serial digestion of pBS+/pci/th1.0 with S1 nuclease and restriction endonuclease, Nde I or Hind III, produces predictable small fragments. Control plasmid are not S1 nuclease sensitive under the experimental conditions employed. This means that the CT_{21}•GA_{21} sequences actually form triple helices. The restriction digest also produces a slightly shorter DNA fragment than is seen for the major restriction product from the pBS+/pci/th1.0 vectors. This suggest that there is another triple helix forming region, at the second pyrimidine rich sequences, +210 to +248.

This novel DNA structure found in intron 1 of the PC gene and flanking DREs may have an inter-relationship with the AhR•Arnt in terms of the PC gene transcriptional regulation. Generally DNA conformational changes accompany DNA interaction with DNA transcription factors thereby producing an enhancement or suppression of gene transcription rates. In the PC gene the triple helix forming DNA sequences may have significant functional role in the mRNA polymerase transcriptional activity. To test this hypothesis, we employed a protein transient expression assay using a wild-type mouse Hepa1c1c7 cell system. Figure 3.8 shows the result of transient expression assay using luciferase reporter vectors. The upstream fragment (1359 bp) of the PC gene has very strong promoter activity in this

Figure 3.7 S1 nuclease assay experimental design and 1.5% (w/v) agarose gel analysis. The pBS$^+$/pci/th1.0 and pBS$^+$ phagmid were subjected to S1 nuclease digestion for 40 minutes as described in experimental procedures section after pre-incubation in 1x reaction buffer containing 30 mM sodium acetate (pH 4.6), 1 mM zinc sulfate and 5% (v/v) glycerol for 30 minutes. S1 nuclease treated DNA was restricted with endonuclease Nde I and Hind III. A 1.5% (w/v) agarose gel electrophoresis was conducted at 40 V of constant voltage for 4 hours to separate the S1 nuclease hypersensitive sites from phagmid following restriction endonuclease treatment. M: 1.0 kb DNA size standard ladder, M*: 100 bp DNA size standard ladder, S: supercoiled phagmid, L: linearized phagmid, 1: Nde I digestion, and 2: Hind III digestion.

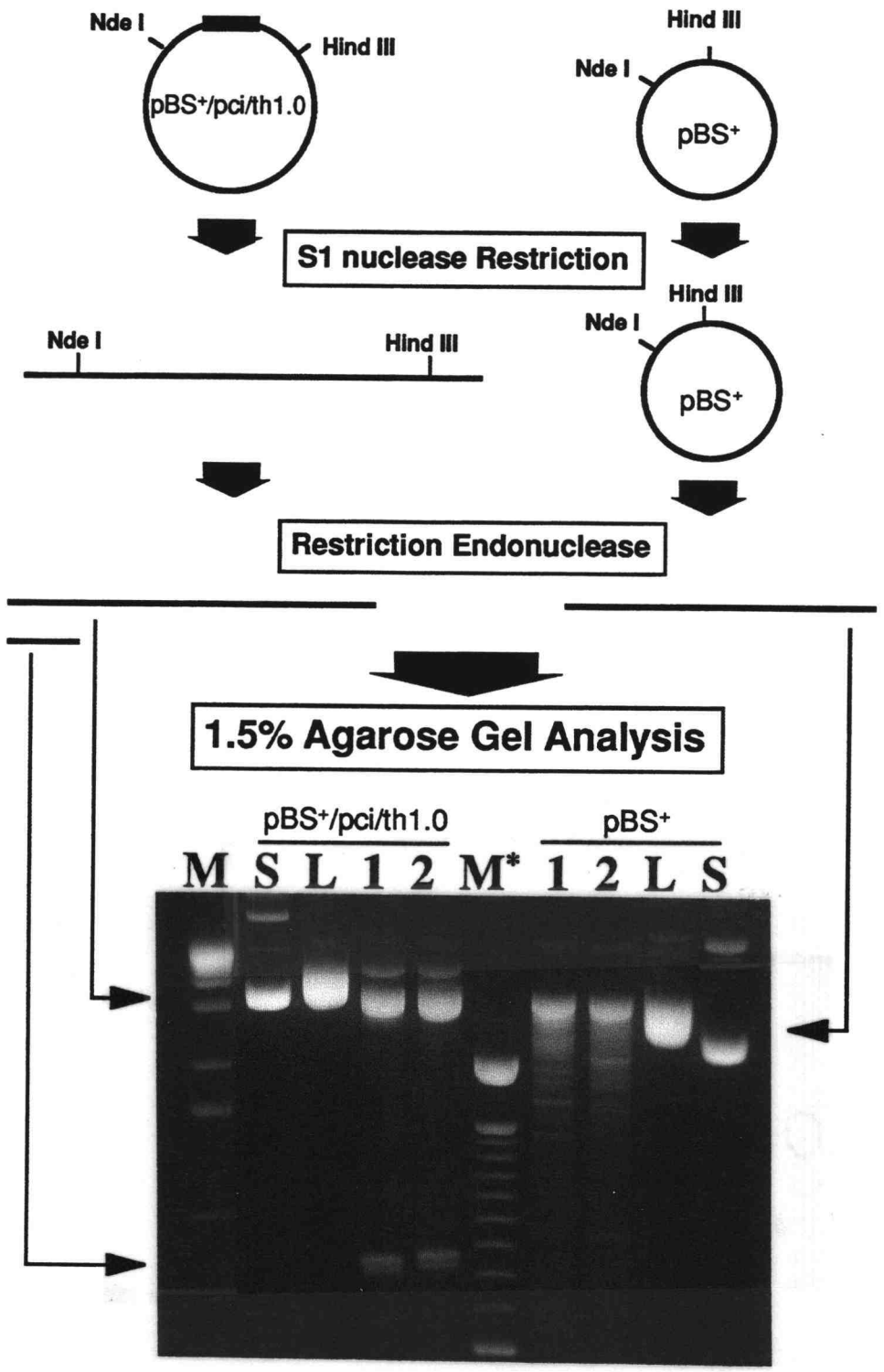
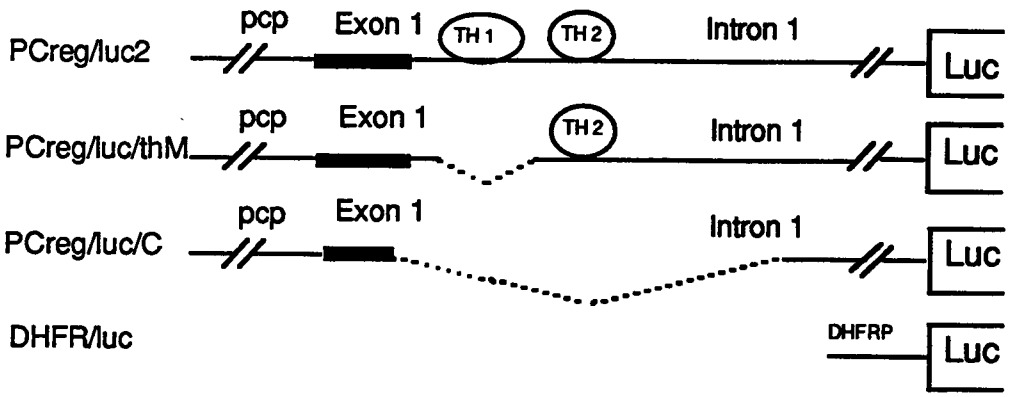

Nde I
Hind III
pBS+/pci/th1.0
Hind III
Nde I
pBS+
S1 nuclease Restriction
Nde I
Hind III
Hind III
Nde I
pBS+
Restriction Endonuclease
1.5% Agarose Gel Analysis
pBS+/pci/th1.0
pBS+
M S L 1 2 M* 1 2 L S

Figure 3.7

Figure 3.8 Constructs of luciferase reporter vectors and relative luciferase activity in Hepa1c1c7. A) PCreg/luc2 contains the 1634 bp upstream promoter, exon 1, and whole intron 1. The PCreg/luc/thM constructed from the PCreg/luc2 by the deletion of the first triple helix forming region (nucleotide position 84 to 250 of intron 1, see Figure 3.6). The PCreg/luc/C was generated from PCreg/luc2 by the deletion of the part of exon 1 and intron 1(from +113 to position 533 nucleotide of intron). The DHFR/luc contains murine dihydrofolate reductase promoter. The PCreg/luc/C is out of open reading for luciferase cDNA because its 5' splicing junction of intron 1 is removed, therefore it used as a control vector for measuring promoter activity of the PC upstream promoter. Using the PCreg/luc/C as a control eliminates the difference of transfection efficiency possibly caused by huge (three times) vector size different between pGL3-Basic and PCreg/luc2. PCP: pyruvate carboxylase promoter (-1359 from transcription start site), TH1 and TH2: the helix forming regions located in +112 to +153 and +210 to +249 nucleotide positions respectively, DHFRP: dihydrofolate reductase gene promoter (-270 to +20). B) Hepa1c1c7 cells (4×10^5 cells/60 x 15 mm dish) were transiently transfected with 10 ~ 30 μg of the reporter vectors and 30 μg of polybrene (Aldrich chemical Co.) followed by dimethyl sulfoxide (DMSO) shock, and luciferase activity was measured as described in experimental procedures. The activity was expressed in relative luminescence units (RLU) per microgram of protein. The *bar graph* shows the mean and standard deviation for minimum three independent DNA transfection experiments for Hepa1c1c7.

A)


PCreg/luc2
pcp
Exon 1
TH 1
TH 2
Intron 1
Luc
PCreg/luc/thM
pcp
Exon 1
TH 2
Intron 1
Luc
PCreg/luc/C
pcp
Exon 1
Intron 1
Luc
DHFR/luc
DHFRP
Luc

B)

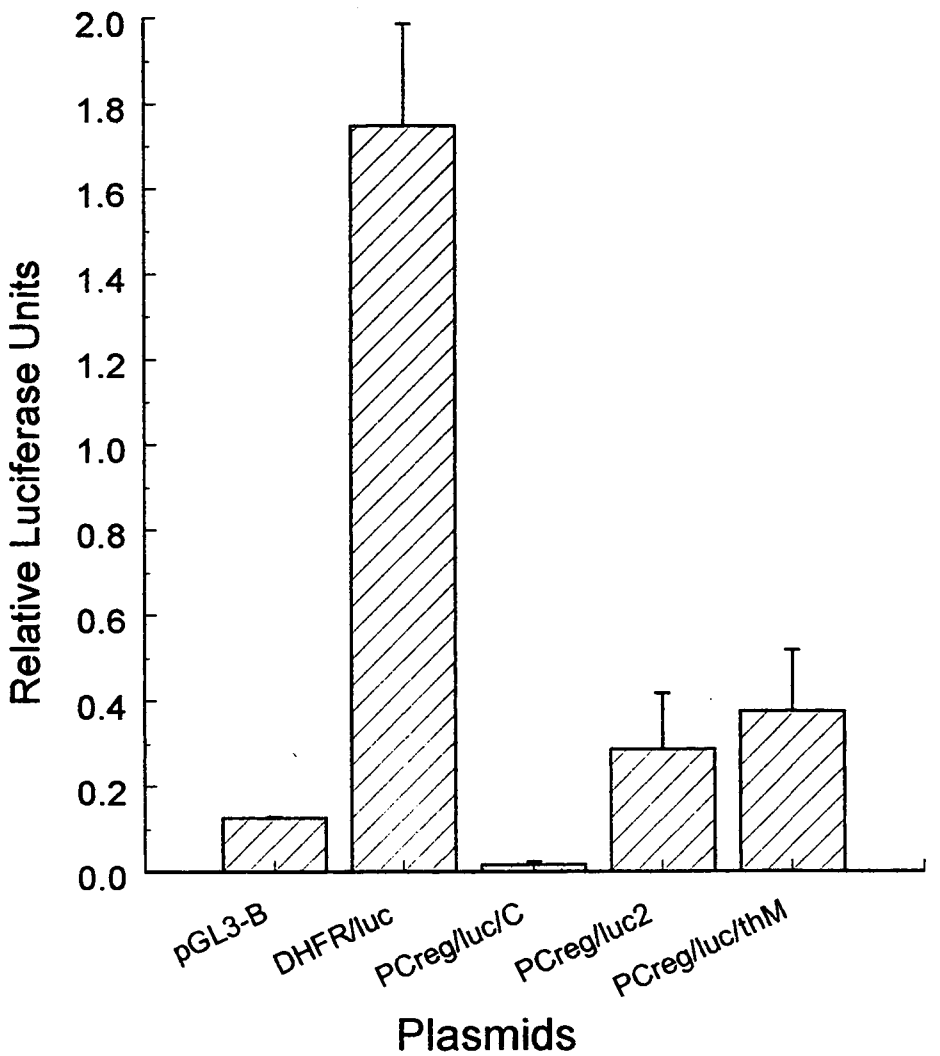

Relative Luciferase Units
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pGL3-B
DHFR/luc
PCreg/luc/C
PCreg/luc2
PCreg/luc/thM
Plasmids

Figure 3.8

experiment, showing roughly 18-fold increased luciferase activity relative to PCreg/luc/C which served as a control. DHFR promoter containing vector, DHFR/luc, used for promoter activity comparison with the promoter of the PC gene, shows 14-fold increased luciferase activity relative to control vector pGL3-B. This strong promoter activity of the PC gene upstream region was conformed another transient transfection experiment using short upstream fragment (792 bp) of the PC gene (Figure 3.3). The triple helix forming region deletion mutant, PCreg/luc/thM, has slightly increased luciferase activity compared to Pcreg/luc but statistically there is no significant difference between those two vectors. This results indicate that the triple helix forming CT_{21}•GA_{21} sequences of the PC gene may not involved in transcriptional regulation.

3.5 Discussion

We have studied the functional role of the upstream and downstream regions of PC gene with regard to the transcriptional regulation of the gene in response to TCDD exposure. In animal studies, TCDD suppresses the PC gene expression and the Ah receptor is required for the effect (Sparrow *et al* 1994; Ilian *et al* 1996; Ryu *et al* 1995). These observations introduce the necessity of evaluating the molecular mechanism involved in the PC gene promoter response to TCDD treatment.

We first cloned the PC gene upstream region using inverse PCR (Ochman *et al* 1990). DNA sequence data obtained for the PC gene upstream region (Figure 3.2) reveals that the PC gene is one of many TATA-less genes characterized by the presence of a functional transcription initiator elements (Inr). The mechanism of transcription initiation at promoters that lack a TATA box , and in particular the role of TATA box

binding protein (TBP), are still poorly characterized. However, it is known that an initiator DNA sequence (Inr) can function as an independent core promoter element which specifies the position of a transcription start site in TATA-less promoters (Javahery *et al* 1994). An Inr binding protein (TFII-I), comparable to TFII-D (binds TATA box) supports basal transcription from an adenovirus major late promoter (Roy *et al.* 1991). Generally, TATA-less promoter containing genes have multiple transcription initiation sites and are characteristic of many house-keeping genes. These facts imply that the PC gene in mouse liver has more than one transcription initiation site, one of which is identified in this report was characterized by B. Sparrow (personal communication) (Figure 3.2). This is consistent with the a recently discovered five alternative forms of rat pyruvate carboxylase cDNAs identified in liver, kidney, brain, and adipose tissue. These are expressed in a tissue specific manner. Additionally, two alternative forms of human PC cDNA have been identified in liver. These PC cDNAs have a common coding region but differ in their 5' untranslated regions (5'UTR) (Jitrapakdee *et al.* 1996).

The functional promoter activity of the PC promoter containing an Inr-like initiator element was clearly demonstrated in this study using transient transfection experiments (Figure 3.3 and Figure 3.8). The upstream fragments of the PC gene shows promoter activity at least as strong as that of dihydrofolate reductase gene which is another constitutively expressed and TATA-less promoter containing gene (McGrogan *et al.* 1985). A TATA-less but Inr containing promoter of murine terminal deoxynucleotidyl transferase (TdT), as reported, is sufficient to promote low levels of specific transcription *in vitro* (Smale and Baltimore 1989). There is little RNA polymerase II processivity conferred by Inr and there is probably a need for

additional transcription factors transactivators which stimulate elongation by RNA polymerase II. This cooperative interaction between Inr binding protein TFII-I and a basic helix-loop-helix transactivator USF (c-myc family) in adenovirus major late promoter was well illustrated in a report by Roy *et al.* (Roy *et al.* 1991). One interesting fact is that TFII-I and transactivator a USF bind to USF recognition motif E-box sequence (CACGTG) TFII-I and USF interact cooperatively in both Inr and E-box binding site. This suggests that these proteins may be structurally related, and possibly in the same family. Another basic helix-loop-helix family protein c-myc specifically recognizes and binds E-box motifs. There is evidence that the myc protein regulates hepatic glycolysis in transgenic mice over-expressing myc (Valera *et al.* 1995). Overexpression of the c-myc under control of a phosphoenolpyruvate carboxykinase gene promoter in transgenic mice induces by three-fold the L-type pyruvate kinase mRNA, increased amounts of 6-phosphofructo-2-kinase gene and liver glucokinase. Considering the inter-relationship among TFII-I, USF, and myc proteins with regard to their functional ability to regulate of gene expression by increasing RNA polymerase II processivity in Inr containing genes, it is conceivable that an Arnt protein, another basic helix-loop-helix transcription factor, may affect PC gene's expression via an E-box interaction. This hypothesis was supported in our experiment shown in Figure 3.3. The overexpression of human Arnt in hepa1c1c7 cell reduced luciferase activity driven by the PC promoter in transient transfection experiment . This may be caused by disruption of Inr and USF cooperation by Arnt. It could also reflect a competition effect with c-myc for E-box motifs which are found in PC gene promoter (five E-box motifs are located within 750 bp upstream form transcription start site).

It was surprising to observe the dose-dependent induction effect of TCDD in an luciferase activity assays driven by the PC promoter in Hepa1c1c7 cells (Figure 3.4). This is particularly notable because DRE or DRE-like sequences were not found in the upstream fragment of PC gene promoter tested (Figure 3.2). The absence of a DRE eliminates the possibility that AhR•Arnt heterodimer can interact directly to with the PC gene promoter. The result may indicate the existence of a novel mechanism AhR mediated signal transduction pathway in alteration of the PC promoter induced activity. One possible scenario to explain the induction effect of TCDD in this experiment is that the Arnt protein acts as a natural repressor interacting with a silencer element located in the PC promoter. Therefore, upon TCDD treatment induces squelching effect pulling Arnt from the PC promoter as a result of hetero-dimerization with AhR. This produces a release of repression. However, the reliability of the scenario is attenuated by a recent report indicating that the concentration of Arnt protein is unaffected by TCDD treatment in wild-type murine Hepa1c1c7 cells (Pollenz 1996). In this report, AhR protein was depleted by 85% after 4 hours of TCDD treatment in wild-type Hepa1c1c7 cells. In contrast, the concentration of Arnt protein remained near a normal level. Overexpression of Arnt in Hepa1c1c7 cells (Figure 3.5) did not abolish the induction effect of TCDD in investigated dose range. The more plausible hypothesis explanation for the induction effect of TCDD on the PC promoter activity likely involves a mechanism which bypasses the AhR and Arnt mediated TCDD signal transduction cascade which is well established in CYP1A1 induction mechanism.

Several lines of evidence indicate a role for Ca^{2+} in the regulation of gluconeogenesis . Every hormone that stimulates gluconeogenesis alters

Ca^{2+} homeostasis. Interruption of the Ca^{2+} distribution in cellular organelles blocks the stimulation of gluconeogenesis. Increased cytosolic free Ca^{2+} levels alone can lead to stimulation of gluconeogenesis (reviewed by Kraus-Friedmann and Feng 1996). Hormones that stimulate gluconeogenesis including glucagon, catecholamines, cAMP, α-adrenergic hormones, and vasopressin increase cytoplasmic calcium concentration ($[Ca^{2+}]_i$) (Williamson and Monck 1989; Kraus-Friedmann 1995). The glucagon- and catecholamine-induced high level of $[Ca^{2+}]_i$ is mediated via cAMP produced by a release of stored Ca^{2+} from endoplasmic reticulum (Staddon and Hansford 1989). α-Adrenergic hormones and vasopressin increase $[Ca^{2+}]_i$ by elevating IP_3 levels in response to hormonal stimulation. This causes an influx of Ca^{2+} into cytoplasm from outside of plasma membrane (Putney and Bird 1993). Activation of Ca^{2+}-dependent protein kinase by a gluconeogenic hormones was suggested in early studies from the laboratory of Garrison (Garrison *et al.* 1984). They report that phosphorylation of many hepatic proteins occurs following administration of gluconeogenic hormone. Mieskes *et al* reported that Ca^{2+}-dependent phosphorylations are due to Ca^{2+}/calmodulin-dependent protein kinase which phosphorylates many proteins (Mieskes 1987). However, there is no study showing that the elevated free intracellular Ca^{2+} and cAMP levels induces increases in mRNA transcription of gluconeogenic enzymes such as pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK). With regard to intracellular Ca^{2+} distribution, it is worth noting that TCDD induces alterations in calcium homeostasis in murine hepatoma cells, guinea pig hepatocytes, and guinea pig papillary muscle (Al-Bayati *et al.* 1988; Canga *et al.* 1988; Puga *et al.* 1992). It is believed that elevation of intracellular Ca^{2+} level by TCDD is caused by liberation of stored calcium from endoplasmic

reticulum as result at inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases. The polycyclic aromatic hydrocarbons (PAHs), a class of compounds structurally related to TCDD and other HAHs, also inhibit sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases. Many PAHs maintain a sustained elevation of intracellular calcium. (Krieger *et al.* 1995; Karras *et al.* 1996). Therefore, it can be postulated that TCDD mimics the effects of gluconeogenic hormones through elevation of intracellular Ca^{2+} and cAMP levels. This yields an activation of Ca^{2+}/calmodulin-dependent protein kinases. Puga *et al.* showed that the first observable effects of TCDD in cultured murine hepatoma cells is a rapid, transient increase in Ca^{2+} influx and significant elevation of activated, membrane-bound protein kinase C. These changes are then followed by induction of the immediate early proto-oncogenes c-fos, jun-B, c-jun, and jun-D, and by large increases in AP-1 transcription factor activity. This induction processes can be blocked by staurosporine, a potent protein kinase C inhibitor. This transcription factor induction effect of TCDD via protein kinase C is in comparable with that of phorbol esters (Puga *et al.* 1992). The results presented in this report (Figure 3.4 and Figure 3.5), the induction effect of TCDD on the PC promoter activity, could be explained by the hypothesis that TCDD deregulates the PC promoter activity by a mechanism mediated by Ca^{2+}/cAMP and protein kinase C (PKC). The proposed signal transduction cascade is shown by schematic drawing (Figure 3.9). The existence of several E-box motifs which are recognized and bound by c-myc in the PC gene promoter (Figure 3.2) and the fact that c-myc protein is a protein that requires phosphorylation to be active support the hypothesis. Additionally the presence of three cAMP response elements which are bound by cAMP response element binding protein (CREB) in the 5' upstream region of the PC gene (Figure 3.2) may

Figure 3.9 Schematic drawing of proposed TCDD induced alteration of gene expression. TCDD mimics gluconeogenic hormones causing an elevated intracellular free calcium level by blocking endoplasmic reticulum membrane bound Ca^{2+}-ATPase. The elevated cytoplasmic Ca^{2+} level stimulates Ca^{2+}/calmodulin dependent protein kinases and plasma membrane bound protein kinase C by phosphorylation causing an activation of MAP-kinase mediated signal transduction cascade and a phosphorylation of many transcription factors including pyruvate carboxylase gene specific transcription factors.

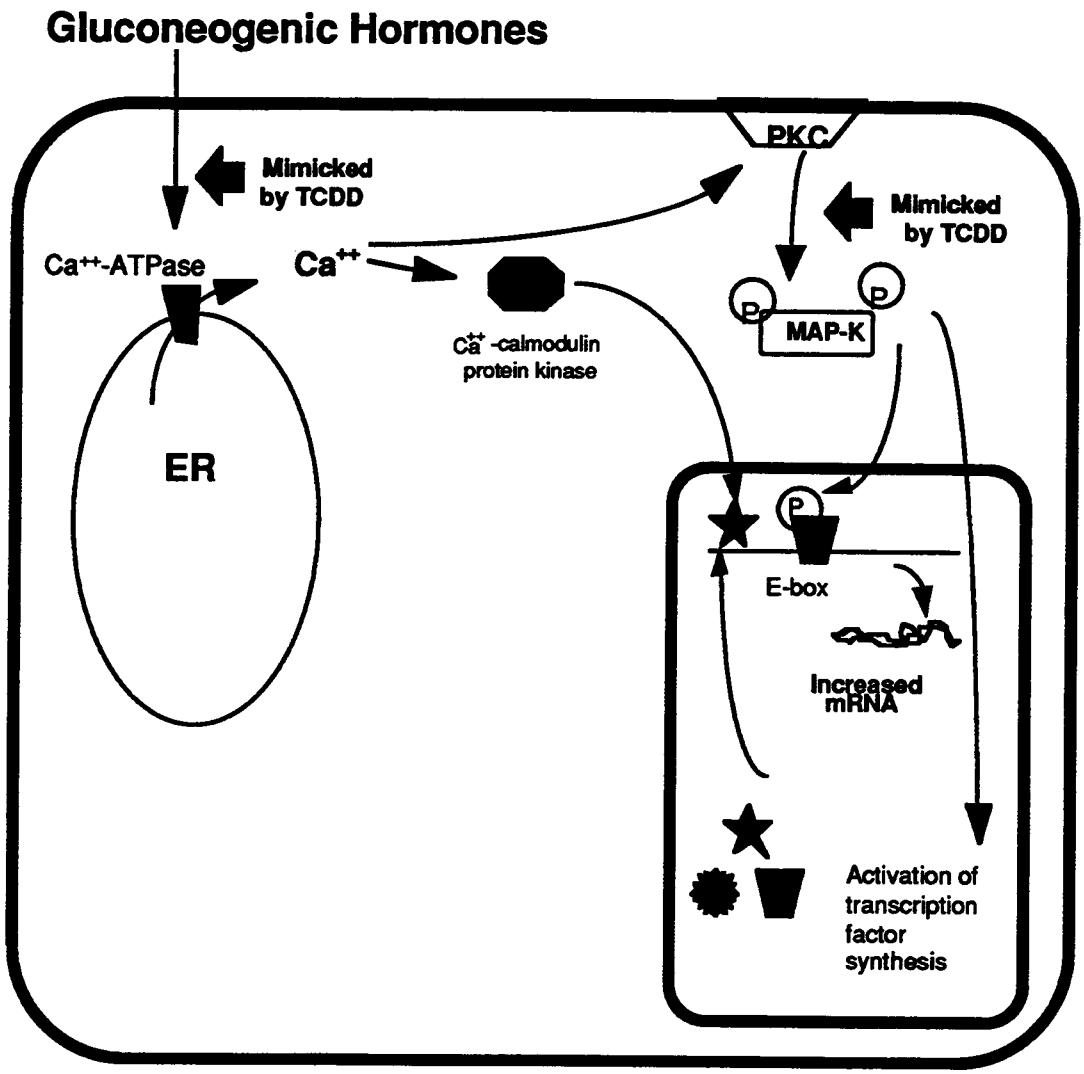

Gluconeogenic Hormones
Mimicked by TCDD
PKC
Mimicked by TCDD
Ca++-ATPase
Ca++
Ca++ -calmodulin protein kinase
MAP-K
ER
E-box
Increased mRNA
Activation of transcription factor synthesis

Figure 3.9

also contribute to increases in the PC promoter activity as a result of TCDD treatment.

Sequence-specific recognition of nucleic acids is essential for the regulation of biological functions including gene replication, transcription, and translation. Although regulation of gene expression is achieved in most cases by specific nucleotide binding proteins, it has been illustrated that nucleic acids could also play a regulatory function (Le Doan *et al.* 1987). They form a local triple helix upon binding of the third oligopyrimidine strand in the major groove of double-stranded DNA in a parallel orientation with respect to the polypurine strand of the double helix (Le Doan *et al.* 1987). The Hoogsteen hydrogen bonding of thymine with A:T and protonated cytosine with G:C Watson-Crick base pairs is used to achieve the triple helical structure (Strobel and Dervan 1990). Polypyrimidine/polypurine tracts have been observed in the vicinity of promoters and within the coding regions of many genes such as *c-myc*, *Drosophila hap 26* and *hsp 70*, SV 40 late genes and b-globin genes (Palecek 1991; Birnboin *et al.* 1979). The structural role of polypyrimidine/polypurine sequences potential to adopt triple helical DNA structure in various biological processes has been suggested (Palecek 1991) but the precise role and mechanism by which these sequences function is not understood. In our experiments, we have shown that PC gene has novel DNA sequences located in the 5' region of intron 1 of the PC gene (Figure 3.6). We have utilized S1 nuclease restriction assay to prove that the polypyrimidine and polypurine (TC_{21}•AG21) and pyrimidine rich sequences adopt the triple helical structure (Figure 3.7). Subsequently, we attempt to analyzed the functional role of the triple helix using transient transfection assay in Hepa1c1c7. The significant functional role of the homopyrimidine/homopurine sequences was not detected by

transient transfection system (Figure 3.8). In contrast, Rando *et al.* reported that insertion of a 45 base-pair purine-rich fragment into the *gag* gene and downstream of bacterial T7 and T3 promoter inhibits the T7 and T3 RNA polymerase directed transcription elongation by northern blot assay (Rando *et al.* 1994). Different experimental methods may account for the contrasting results. The polypyrimidine/polypurine sequences located in upstream and down stream promoter regions, with the potential to form a triple helical DNA structure have been implicated to have a crucial functions in the transcription of various genes. For example, the -125 region of *c-myc* gene forming intramolecular triple helix structure is clearly involved in the regulation of the gene transcription. The deletion of the triple helix forming region reduced the expression of the gene (Strobel and Dervan 1990).

Eukaryotic gene expression in *vivo* is regulated in nucleosomal template, in which the higher order structure of DNA is adopted. To be active, any gene's regulatory sequences and promoter/transcription start site must be located in nucleosome free domain-an accessible nonnucleosomal conformation (Travers 1992). Such a nonnucleosomal site can be detected as a DNase 1-hypersensitive site (Gross and Garrard 1988). It is suggested that many inducible genes may be classified as one of two types, a remodeling gene or preset gene. In remodeling gene, some regulatory sequences such as the TATA box or other transcription factor binding sites are blocked by nucleosomes when the gene is in an inactive state. During gene activation, the nucleosomal positions are remodeled to expose DNA to transcription factors. In contrast, regulatory regions of a preset gene are in nucleosome free domains and are maintained in an accessible conformation while the gene is inactive. Therefore, in a preset gene, remodeling of the nucleosomal pattern is not required before or after

gene activation. Such a gene must be packaged into precise chromatin structure immediately after replication. Qin Lu *et al.* demonstrated the activating ability of $(CT)_n$:$(GA)_n$ sequences located in a promoter region of *Drosophila hsp 26* gene (Qin Lu *et al.* 1993). In this report, they found that the GAGA factor (an abundant transcription factor) is required for normal expression of many *Drosophila* genes. when it is bound to a target region, it establishes and/or maintains the DNase 1-hypersensitive sites. Therefore we propose that the $(TC)_{21}\bullet(AG)_{21}$ homopyrimidine region found in PC gene 5' side intron 1 (Figure 3.6) may have the similar function in stabilizing precisely preset nucleosomal conformation and/or remodeling nucleosomal structure in the region of the PC gene regulatory regions. It is worthwhile to note that a putative DRE sequence found in intron 1 is flanked by two polypyrimidine/polypurine regions (Figure 3.6). Thus, it will be interesting to see if there is any inter-relationship between the polypyrimidine sequence binding GAGA factor and TCDD induced AhR:Arnt heterodimer with respect to the nucleosomal conformation alteration and transcription factor accessibility change in regulation of the PC gene expression. Such involvement of nucleosomal structure in regulation of gene expression may be a factor that contributes the results of different responses of PC gene with TCDD treatment in animal experiment (Sparrow *et al* 1994; Ryu *et al.* 1995; Ilian *et al.* 1996, and Chapter 2 of this thesis) and cell culture experiments using transient transfection (Figure3.4 and Figure 3.5). In the transient expression experiments, the vector DNA is not replicated and it does not receive a full histone compliance.

3.6 References

Al-Bayati, Z. A. F., Murray, W. J., Pankaskie, M. C., and Stohs, S. J. (1988). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced perturbation of calcium distribution in the rat. *Res. Commun. Chem. Pathol. Pharmacol.* **60:** 47-56.

Antonsson, C., Arulampal, V., Whitelaw, M. L., Pettersson, S., and Poellinger, L. (1995). Constitutive function of the basic helix-loop-helix/PAS factorArnt. *J. Biol. Chem.* **270:** 13968-13972.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. H., and Struhl, K. (1991). Direct DNA sequencing of PCR products. *In Current Protocols in Molecular Biology, Volume 2*, Greene Publishing Associates & Wiley-interscience: 15.2.1-15.2.5.

Bergot, M. -O., Diaz-Guerra, M. -J., Puzenat, N., Raymondjean, M., and Kahn A. (1992). Cis-regulation of L-type pyruvate kinase gene promoter by glucose, insulin and cyclic AMP. *Nucleic Acid s Res.* **2:** 1871-1878.

Biggin, M. D., and Tjian, R. (1988). Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. *Cell.* **53:** 699-711.

Birnboin, H. C., Sederoff, R. R., and Paterson, M. C. (1979). Distribution of polypyrimidine • polypurine segments in DNA from diverse organisms. *Eur. J. Biochem.* **98:** 301-307.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **72:** 248-558.

Burbach, K. M., Poland, A., and Bradfield, C. A., (1992). Cloning of Ah receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89:** 8185-8189.

Canga, I., Levi, R., and Rifkind, A. B. (1988). Heart as a target organ in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity: Decreased b-adrenergic responsiveness and evidence of increased intracellular calcium. *Proc. Natl. Acad. Sci. USA.* **85:** 905-912.

Cuif, M. H., Porteu, A., and Kahn, A. (1993). Exploration of a liver-specific glucose/insulin-responsive promoter in transgenic mice. *J. Biol. Chem.* **168:** 13769-13772.

Dang, C. V., Dolde, C., Gillison, M. L., and Kato, G. J. (1992). Discrimination between related DNA site by a single amino acid residue of myc-related basic-helix-loop-helix proteins. *Proc. Natl. Acad. Sci. U. S. A.* **89:** 599-602.

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

Dunn, T.J., Lindahl, R. and Pitot, H.C. (1988). Differential gene expression in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): noncoordinate regulation of a TCDD-induced aldehyde dehydrogenase and cytochrome P-450c in the rat. *J. Biol. Chem.* **263:** 10878-10886.

Ema, M., Sogawa, K., Watanabe, N., Chukoh, Y., Matsushita, N., Gotoh, O., Funae, Y., and Fujii-Kuriyama, Y. (1992). cDNA cloning and structure of mouse putative Ah receptor. *Biochem. Biophys. Res. Commun.* **184:** 246-253.

Evans, T. and Efstratiadis, A. (1986). Sequence-dependent S1 nuclease hypersensitivity of a heteronomous DNA duplex. *J. Biol. Chem.* **261:** 14771-14780.

Garrison, J. C., Johnsen D. E., and Campanile, C. P. (1984). Evidence for the role of phosphorylase kinase, protein kinase C and other Ca^{2+} -sensitive protein kinase in the responses of hepatocytes to angiotensin II and vasopresin. *J. Biol. Chem.* **259:** 3283-3292.

Gross, D. S., and Garrard, W. T. (1988). Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* **57:** 159-197.

Hoffman, E. C., Reues, H., Chu, F. F., Sander, F.,Conley, N. H.,Brooks, B. A., and Hankinson, O., (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252:** 954-958.

Hujisawa-Sehara, A., Sogawa, K., Uamane, M., and Fujii-Kuriyama, Y. (1988). A DNA-binding factor specific for the xenobiotic responsive elements of P-450c gene exists as a cryptic form in cytoplasm: its possible translocation to nucleus. *Proc. Natl. Acad. Sci. USA* **85:** 5859-5863.

Ilian, M. A., Sparrow, B. R., Ryu, B. -W., Selivonchick, D. P., and Schaup, H. W. (1996). Expression of hepatic pyruvate carboxylase mRNA in C57BL/6J $Ah^{b/b}$ and congenic $Ah^{d/d}$ mice exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J. Biochem. Toxicol.* **11**:51-56.

Jaiswal, A.W. (1991). Human NAD(P)H: quinone oxidoredutase (NQO_1) gene structure and induction by dioxin. *Biochemistry* **30:** 10647-10653.

Javahery, R., Khachi, A., Lo, K., Zenzie, G. B., and Smale, S. T. (1994). DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol. Cell. Biol.* **14:** 116-127.

Jitrapakdee, S., Walker, M. E., and Wallace, J. C. (1996). Identification of novel alternatively spliced pyruvate carboxylase mRNAa with divergent 5'-untranslated regions which are expressed in a tissue specific manner. *Biochem. Biophys. Res. Comm.* **223:**695-700.

Karras, J. G., Morris, D. L., Matulka, R. A., Kramer, C. M., and Holsapple, M. P. (1996). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elevates basal B-cell intracellular calcium concentration and suppresses surface Ig- but not CD40-induced antibody secretion. *Toxicol. Appl. Pharmacol.* **137:** 275-284.

Kawai, S. and Nishizawa, M. (1984). New procedure for DNA transfection with polybrene and dimethyl sulfoxide. *Mol. Cell. Biol.* **4:** 1172-1174.

Kraus-Friedmann, N,. and Feng L. (1996). The role of intracellura Ca^{2+} in the regulation of gluconeogenesis. *Metabolism* **45:** 389-403.

Kraus-Friedmann, N. (1995). Hormonal regulation of cytosolic calcium levels in liver. *Brazil J. Med. Biol. Res.* **28:** 275-284.

Krieger, J. A., Davila, D. R., Lytton, J. L., Born, J. L., and Burchiel, S. W. (1995). Inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) by polycyclic aromatic hydrocarbons in HPB-ALL human T cells and other tissues. *Toxicol. Appl. Pharmacol.* **133:** 102-108.

Lamaigre, F. P. and Rousseau, G. G. (1994). Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult rat liver. *Biochem. J.* **303:** 1-14.

Le Doan, T., Perrouault, D., Praseuth, D., Habhoub, N., Decount, J. L., Thuong, N. T., Lhomme, J., and Helene, C. (1987). Sequence-specific recognition, photocrosslinking and cleavage of DNA double helix by an oligo-[α]-thymidylate covalently linked to an azidpproflavine derivative. *Nucleic Acids Res.* **15:** 7749-7760.

Liu, Z., Thompson, K. S., and Towle, H. C. (1993) Carbohydrate regulation of the rat L-type pyruvate kinase gene requires two nuclear facyors. *J. Biol. Chem.* **26:** 12787-12795.

Max, S.R., and Silberlgeld, E.K. (1987). Skeletal muscle glucocorticoid receptor and glutamine synthetase activity in the wasting syndrome in rats treated with 2, 3, 7, 8 -tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmcol.* **87:** 523-527.

McGrogan, M., SImonsen, C. C., Smouse, D. T., Farnham, P. J., and Schimke, R. T. (1985). Heterogeneity at the 5' terminal of mouse dihydrofolate reductase mRNA. *J. Biol. Chem.* **260:** 2307-2314.

Mieskes, G., Kuduz, J., and Soling, H-D. (1987). Are calcium-dependent protein kinase involved in the regulation of glycolytic/gluconeogenic enzymes? *Eur. J. Biochem.* **167:** 383-389.

Morgan, J. E. and Whitlock, J. P. Jr. (1992). Transcription-dependent and transcription-independent nucleosome disruption induced by dioxin. *Proc. Natl. Acad. Sci. USA* **89:** 11622-11626.

Nambu, J. R., Lewis, J. O., Wharton, K. A. Jr., and Crews, S. T. (1991). The drosophilar single-minded gene encodes a helix-loop-helix protein that act as a master regulator of CNS midline development. *Cell* **20:** 1157-1167

Nebert, D.W., and Gonzalez, F.J. (1987). P450 genes: structure, evolution, and regulation. *Annu. Rev. Biochem.* **56:** 945-993.

Ochman, H., Medhora, M. M., Garza, D., and Hartl, D. L. (1990). Amplication of flanking sequences by inverse PCR. *In PCR protocols: a guide to methods and amplications*, Innis, M. A., Gelfanf, D. H., Sninsky, J. J., and White, T. J. eds., Acdemic Press: 219-227.

Owens, I.S. (1977). Genetic regulation of UDP-glucuronyltransferase induction by polycyclic aromatic compounds in mice: co-segregation with aryl hydrocarbon hydroxylase induction. *J. Biol. Chem.* **252:** 2827-2833.

Palecek, E. (1991). Local supercoil-stabilized DNA structures. *Critical. Rev. Biochem. Mol. Biol.* **26:** 151-226.

Panyutin, I. G. and Wells, R. D. (1991). Nodule DNA in the $(GA)_{37} \bullet (CT)_{37}$ insert in superhelical plasmids. *J. Biol. Chem.* **267:** 5495-5501.

Perdew, G. H. (1988). Association of the Ah receptor with the 90-kDa heat shock protein. J. Biol. Chem. 263: 13802-13805.

Peterson, R.E., Seefeld, M.D., Christian, B.J., Potter, C.L., Kellin, C.K., and Keesey, R.E. (1984). The wasting syndrome in 2, 3, 7, 8

-tetrachlorodibenzo-p-dioxin toxicity: Basic features and their interpretation. *Banbury Report* **18:** 291-308.

Pollenz, R. S. (1996). The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Pharmacol. Exp. Therapeutics* **49:** 391-398.

Puga, A., and Nebert, D. W., and Carrier, F. (1992). Dioxin induces expression of c-fos and c-jun proto-oncogenes and a large increase in transcription factor AP-1. *DNA Cell Biol.* **11:** 269-281.

Putney, J. W. and Bird, G. S. L. (1993). The inositol phosphate-calcium signaling system in non-excitable cells. *Endor. Rev.* **14:** 610-631.

Qin Lu, Wallrath, L. L., Granok, H., and Elgin. S. C. R. (1993). $(CT)_n$:$(GA)_n$ repeats and heat shock elements have distinct roles in chromatin structure and transcritpional activation of the *Drosophila hsp26* gene. *Mol. Cell. Biol.* **13:** 2802-2814.

Rando, R. F., DePaolis, L., Durland, R. H., Jayaraman, K., Kessler, D. J., and Hogan M. E. (1994). Inhibition of T7 and T3 RNA polymerase directed transcription elongation *in vitro. Nucleic Acid Res.* **22:** 678-685.

Roeder, R. G. (1996). The role of general initiation factors in transcription by RNA polymerase II. *Trends in Biochem. Sci.* **21:** 327-335

Roy, A. L., Meisteremst, M., Pognonec, P., and Roeder. (1991). Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. *Nature* **354:** 245-248.

Rushmore, T.H. and Pickett, C.B. (1990). Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene: characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J. Biol. Chem.* **265:** 14648-14653.

Ryu, B. -W., Shukla, R., Sparrow, B. R. Selivonchick, D. P., and Schaup, H. W. (1995). Ah receptor involvement in mediation of pyruvate carboxylase levels and activity in mice given 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biochem. Toxicol.* **10:** 103-109.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Identification of bacterial colonies that contain recombimant plasmids. *In Molecular Biology, a laboratory manual, 2nd edition,* Cold Spring Harbor Laboratory Press: 1.85-1.86.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Strategies for cloning in plasmid vectors. *In Molecular Biology, a laboratory manual, 2nd edition,* Cold Spring Harbor Laboratory Press: 1.53-1.84.

Scheuplein, R. J., Gallo, M. A., and van der Heijden, D. A. (1991). Epilogue, *banbury Report* **35:** 489-490.

Shih, H.-M. and Towle, H. C. (1992). Definition of the carbohydrate response element of the rat S14 gene. *J. Biol. Chem.* **267:** 13222-13228.

Singleton, C. K., Klysik, J., Stirdivant, S. M., and Wells, R. D. (1982). Left-handed Z-DNA is induced by supercoiling in physiologic ionic condition. Nature 299: 312-316.

Smale, S. T. and Baltimore, D. (1989). The "Initiator" as a transcription control element. *Cell* **57:** 103-113.

Sparrow, B.R., Thompson, C.S., Ryu, B.-W, Slevonchick, D.P. and Schaup, H.W., 1994. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced alterations of pyruvate carboxylase levels and lactate dehydrogenase isozyme shifts in C57BL/6J male mice. *J. Biochem. Toxicol.* **9:** 329-335.

Staddon, J. M. and Hansford, R. G. (1989). Evidence indicating that the glucagon-induced increases in cytoplasmic free Ca^{2+} concentration in hepatocytes is mediated by increases in cyclic-AMP concentration. *Eur. J. Biochem.* **179:** 47-52.

Stahl, B.U., Beer, D.G., Weber, L.W.D., and Rozman, K. (1993). Reduction of hepatic phosphoeolpyruvate carboxykinase (PEPCK) activity by . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is due to decreased mRNA levels. *Toxicology*, **79:** 81-95.

Strobel, S. A. and Dervan, P. B. (1990). Site-specific cleavage of a yeast chromosome by oligonucleotide-directed triple-helical formation. *Science* **249:** 73-75.

Takahashi, J. S. (1992). Circadian clock genes are ticking. *Science* **258:** 238-240.

Takimoto, R., Lindahl, R. and Pitot, H.C. (1991). Superinduction of 2,3,7,8-tetrachlordibenzo-*p*-dioxin-inducible expression of aldehyde dehydrogenase by the inhibition of protein synthesis. *Biochem. Biophys. Res. Commun.* **180:** 953-959.

Travers, A. A. (1992). The reprogramming of transcriptional competence. *Cell* **69:** 573-575.

Valera. A., Pujol, A., Gregori, X., Riu, E., Visa, J., and Bosh F. (1995). Evidence from transgenic mice that myc regulates hepatic glycolysis. *FASEB* **9:** 1067-1078.

Weber, L.W.D., Greim, H., and Rozman, K. (1987). Metabolism and distribution of [^{14}C]glucose in rats treated with 2,3,7,8-etrachlorodibenzo-*p*-dioxin (TCDD). *J. Toxicol. Environ. Health* **22:** 195-200.

Weber, L.W.D., Lebofsky, M., Greim, H., and Rozman, K. (1991). Key enzymes of gluconeogenesis are dose-dependently reduced in . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Arch. Toxicol.* **65:** 119-123.

Whitelaw, M. L., Gustafsson, J. A., and Poellinger, L. (1994). Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor Arnt: inducible versus constitutive modes of regulation. Mol. Cell. Biol. 14: 8343-8355.

Williamson, J. R. and Monck, J. R. (1989). Hormone effects on cellular Ca^{2+} fluxes. *Annu. Rev. Physiol.* **51:** 107-124

Zhang, J., Xia, W. -L., Brew, K., and Ahmad, F. (1993). Adipose pryuvate carboxylase; Amino acid sequence and domain structure deduced cDNA sequencing. *Proc. Natl. Acad. Sci. USA.* **90:** 1766-1770.

Bibliography

Al-Bayati, Z. A. F., Murray, W. J., Pankaskie, M. C., and Stohs, S. J. (1988). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced perturbation of calcium distribution in the rat. *Res. Commun. Chem. Pathol. Pharmacol.* **60:** 47-56.

Antonsson, C., Arulampal, V., Whitelaw, M. L., Pettersson, S., and Poellinger, L. (1995). Constitutive function of the basic helix-loop-helix/PAS factorArnt. *J. Biol. Chem.* **270:** 13968-13972.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. H., and Struhl, K. (1991). Direct DNA sequencing of PCR products. *In Current Protocols in Molecular Biology, Volume 2*, Greene Publishing Associates & Wiley-interscience: 15.2.1-15.2.5.

Barney, R. S., Cynthia, T. S., Ryu, B. -W., Selevonchick, D. P., and Schaup, H. W. (1994). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced alteration of pyruvate carboxylase level and lactate dehydrogenase isozyme shifts in C57BL/6J male mice. *J. Biochem. Toxicol.* **9:** 329-335.

Barsotti, D.A., Abrahamson, L.J. and Allen, J.R. (1979). Hormonal alterations in female rhesus monkeys fed a diet containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *Bull. Environ. Contam. Toxicol.* **211:** 463-469

Benington, P. (1969). Data reduction and error analysis in the physical *sciences,* p. 64, McGraw Hill, New York.

Bergot, M. -O., Diaz-Guerra, M. -J., Puzenat, N., Raymondjean, M., and Kahn A. (1992). Cis-regulation of L-type pyruvate kinase gene promoter by glucose, insulin and cyclic AMP. *Nucleic Acid s Res.* **2:** 1871-1878.

Berndt, J., Messner, B, Turkki, T. and Weissmann, E. (1978). Optical assay of pyruvate carboxylase in crude liver homogenates. *Anal. Biochem.* **86:** 154-158.

Biggin, M. D., and Tjian, R. (1988). Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. *Cell.* **53:** 699-711.

Birnbaum, L. (1986). Distribution and excretion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in congenic strains of mice which differ at the Ah locus. *Drug Metabol. Dispos.* **14:** 34-40.

Birnbaum, L. (1991). Developmental toxicity of TCDD and related compounds: Species sensitivities and differences. *Banhury Report* **35:** 51-68.

Birnbaum, L.S., McDonald, M.M., Blair, P.C., Clark, A.M., and Harris, M.W., (1990). Differential toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J mice congenic at the Ah Locus. *Fundam. Appl. Toxicol.* **15:** 186-200.

Bimboin, H. C., Sederoff, R. R., and Paterson, M. C. (1979). Distribution of polypyrimidine • polypurine segments in DNA from diverse organisms. *Eur. J. Biochem.* **98:** 301-307.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **72:** 248-558.

Burbach, K. M., Poland, A., and Bradfield, C. A., (1992). Cloning of Ah receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89:** 8185-8189.

Burbach, K. M., Poland, A., and Bradfield, C. A., (1992). Cloning of Ah receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89:** 8185-8189.

Canga, I., Levi, R., and Rifkind, A. B. (1988). Heart as a target organ in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity: Decreased b-adrenergic responsiveness and evidence of increased intracellular calcium. *Proc. Natl. Acad. Sci. USA.* **85:** 905-912.

Chahoud, I, Krowke, R., Schimmel, A., Merker, H., and Nebert, D. (1989). Reproductive toxicity and pharmacokinetics of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, I. Effects of high doses on the fertility of male rats. *Arch. Toxicol.* **63:** 432-439.

Chomcynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate phenol-chloroform extraction. *Anal. Biochem.* **162:** 156-159.

Couture, L.A., Abbott, B.D., and Birnbaum, L.S. (1990). A critical review of the developmental toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Recent advances toward understanding the mechanism. *Teratology* **42:** 619-632.

Cuif, M. H., Porteu, A., and Kahn, A. (1993). Exploration of a liver-specific glucose/insulin-responsive promoter in transgenic mice. *J. Biol. Chem.* **168:** 13769-13772.

Dang, C. V., Dolde, C., Gillison, M. L., and Kato, G. J. (1992). Discrimination between related DNA site by a single amino acid residue of myc-related basic-helix-loop-helix proteins. *Proc. Natl. Acad. Sci. U. S. A.* **89:** 599-602.

Dannenberg, A.J., Yang, E.K., and Aharon, D. (1983). Dietary lipids induce phase 2 enzymes in rat's small intestine. *Biochem. Biophys. Acta.* **1210:** 8-12.

Demant, P. and Hart, A. (1986). Recombinant congenic strains: a new tool for analyzing genetic traits determined by more than one gene. *Immunogenetics.* **24:** 416-422.

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

DeVito, M. J., and Birnbaum, L. S. (1994). Toxicology of Dioxin and related chemicals. *Dioxin and Health.* Schecter A., Plenum Press: 139-162.

Dewille, J.W., and Farmer, S.J. (1993). Linoleic acid controls neonatal tissue specific stearoyl-CoA desaturase mRNA levels. *Biochem, Biophys. Acta.* **1170:** 291-295.

Dunn, T.J., Lindahl, R. and Pitot, H.C. (1988). Differential gene expression in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): noncoordinate regulation of a TCDD-induced aldehyde dehydrogenase and cytochrome P-450c in the rat. *J. Biol. Chem.* **263:** 10878-10886.

Ema, M., Sogawa, K., Watanabe, N., Chukoh, Y., Matsushita, N., Gotoh, O., Funae, Y., and Fujii-Kuriyama, Y. (1992). cDNA cloning and structure of mouse putative Ah receptor. *Biochem. Biophys. Res. Commun.* **184:** 246-253.

Evans, R., (1988). The steroid and thyroid hormone superfamily. *Science* **240:** 889-892.

Evans, T. and Efstratiadis, A. (1986). Sequence-dependent S1 nuclease hypersensitivity of a heteronomous DNA duplex. *J. Biol. Chem.* **261:** 14771-14780.

Fingerhut, M.A., Halperin, W.E., Marlow, D.A., Piacitelli, L.A., Honchar, P.A., Sweeney, M.H., Griele, A.L., Dill, P.A., Steenland, K., and Suruda, A.H. (1991). Cancer mortality in workers exposed to 2, 3, 7, 8 -tetrachlorodibenzo-*p*-dioxin. *N. Engl. J. Med.* **324:** 212-218.

Garrison, J. C., Johnsen D. E., and Campanile, C. P. (1984). Evidence for the role of phosphorylase kinase, protein kinase C and other Ca^{2+} -sensitive protein kinase in the responses of hepatocytes to angiotensin II and vasopresin. *J. Biol. Chem.* **259:** 3283-3292.

Gonzalez, F.J., Tukey, R.H., and Nebert, D.W. (1984). Structural gene products of the Ah locus. Transcriptional regulation of cytochrome P1450 and P3450 mRNA levels by 3-methylchloanthrene. *Mol. Pharmacol.* **26:** 117-121.

Gross, D. S., and Garrard, W. T. (1988). Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* **57:** 159-197.

Hames, B.D. and Rickwood, D. (1990). One dimensional polyacrylamide gel electrophoresis. In Gel Electrophoresis of Proteins: A Practical Approach, 2 nd ed., Hames, B.D., and Richwood, D., eds., pp. 6-50, Oxford Press, New York.

Henck, J. W., New, M. A., Kociba, R. J., and Rao, K. S. (1981). 2,3,7,8-tetrachlorodibenzo-p-dioxin: Acute oral toxicity on hamsters. *Toxicol. Appl. Pharmacol.* **59:** 405-407.

Hoffman, E. C., Reues, H., Chu, F. F., Sander, F.,Conley, N. H.,Brooks, B. A., and Hankinson, O., (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252:** 954-958.

Hujisawa-Sehara, A., Sogawa, K., Uamane, M., and Fujii-Kuriyama, Y. (1988). A DNA-binding factor specific for the xenobiotic responsive elements of P-450c gene exists as a cryptic form in cytoplasm: its possible translocation to nucleus. *Proc. Natl. Acad. Sci. USA* **85:** 5859-5863.

Ilian, M. A., Sparrow, B. R., Ryu, B. -W., Selivonchick, D. P., and Schaup, H. W. (1996). Expression of hepatic pyruvate carboxylase mRNA in C57BL/6J $Ah^{b/b}$ and congenic $Ah^{d/d}$ mice exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biochem. Toxicol.* **11:**51-56.

Isarel, D.I. and Whitlock, Jr. J.P., 1983. Induction of mRNA specific for cytochrome P_1-450 in wild type and variant mouse hepatoma cells. *J. Bio. Chem.* **258:** 110390-10394.

Isarel, D.I., and Whitlock, Jr. J.P., 1984. Regulation of cytochrome P_1-450 gene transcription by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in wild type and variant mouse hepatoma cells. *J. Biol. Chem.* **259:** 5400-5402.

Jaiswal, A.K. (1991). Human NAD(P)H: quinone oxidoreductase (NQQ_1) gene structure and induction by dioxin. *Biochemistry* **30:** 10647-10653.

Javahery, R., Khachi, A., Lo, K., Zenzie, G. B., and Smale, S. T. (1994). DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol. Cell. Biol.* **14:** 116-127.

Jitrapakdee, S., Walker, M. E., and Wallace, J. C. (1996). Identification of novel alternatively spliced pyruvate carboxylase mRNAa with divergent 5'-untranslated regions which are expressed in a tissue specific manner. *Biochem. Biophys. Res. Comm.* **223:**695-700.

Jones, K.G., and Sweeney, G.D. (1980). Dependence of the porpyrogenic effect of . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin upon inheritance of arylhydrocarbon hydroxylase responsiveness. *Toxicol. Appl. Pharmacol.* **87:** 42-49.

Jones, M.K., Weisenburger, W.P., Sipes, I.G.and Russel, D.H. (1987). Circadian alterations in prolactin, corticosterone, and thyroid hormone levels and down-regulation of prolactin receptor activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* **87:** 337-350.

Karras, J. G., Morris, D. L., Matulka, R. A., Kramer, C. M., and Holsapple, M. P. (1996). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elevates basal B-cell intracellular calcium concentration and suppresses surface Ig- but not CD40-induced antibody secretion. *Toxicol. Appl. Pharmacol.* **137:** 275-284.

Kawai, S. and Nishizawa, M. (1984). New procedure for DNA transfection with polybrene and dimethyl sulfoxide. *Mol. Cell. Biol.* **4:** 1172-1174.

Kerkvliet, N. (1994). Immunotoxicology of Dioxin and Related Chemicals. *Dioxin and Health.* Schecter A.,Plenum Press: 139-162.

Knutson, J.C., and Poland, A. (1982). Response of murine epidermis to . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: interaction of Ah and hr loci. *Cell.* **30:** 225-234.

Kociba, R.J., Keller, P.A., Park, C.N. and Gehring, P.J. (1976). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD): Results of a 13-week oral toxicity study in rats. *Toxciol. Appl. Pharmacol.* **35:** 553-574.

Kraus-Friedmann, N,. and Feng L. (1996). The role of intracellura Ca^{2+} in the regulation of gluconeogenesis. *Metabolism* **45:** 389-403.

Kraus-Friedmann, N. (1995). Hormonal regulation of cytosolic calcium levels in liver. *Brazil J. Med. Biol. Res.* **28:** 275-284.

Krieger, J. A., Davila, D. R., Lytton, J. L., Bom, J. L., and Burchiel, S. W. (1995). Inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) by polycyclic aromatic hydrocarbons in HPB-ALL human T cells and other tissues. *Toxicol. Appl. Pharmacol.* **133:** 102-108.

Kumaki, K., Jensen, N.M., Shire, J.G.M., and Nebert, D.W. (1977). Genetic differences in induction of cytosol reduced-NADPH: menadione oxidoreductase and microsomal arylhydrocarbon hydroxylase in the mouse. *J. Biol. Chem.* **252:** 157-165.

Lamaigre, F. P. and Rousseau, G. G. (1994). Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult rat liver. *Biochem. J.* **303:** 1-14.

Le Doan, T., Perrouault, D., Praseuth, D., Habhoub, N., Decount, J. L., Thuong, N. T., Lhomme, J., and Helene, C. (1987). Sequence-specific recognition, photocrosslinking and cleavage of DNA double helix by an oligo-[α]-thymidylate covalently linked to an azidpproflavine derivative. *Nucleic Acids Res.* **15:** 7749-7760.

Lentnek, M., Griffith, O.W., and Rifkind, A.B. (1991). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin increases reliance on fats as a fuel source independently of diet: Evidence that diminished carbohydrate supply contributes to dioxin lethality. *Biochem. Biophy. Res. Comm.* **174:** 1267-1271.

Liu, Z., Thompson, K. S., and Towle, H. C. (1993) Carbohydrate regulation of the rat L-type pyruvate kinase gene requires two nuclear facyors. *J. Biol. Chem.* **26:** 12787-12795.

Mackay, D., Shiu, W.-Y., and Ma, K. (1992). *Illustrated hand book of Physical -chemical properties and Environmental Fate of Organic chemicals Volume II*, Lewis Publishers, Chelsea. MI.

Martin, B., Marchaland, C., Phillips, J., Chapouthier, G., Spach, C., and Motta, R., 1992. Recombinant congenic strains of mice from B10.D2 and DBA/2: their contribution to behavior genetic research and application to audiogenic seizures. *Behavior Genetics.* **22:** 685-701.

Martin, G., Vincent, N., Combet, C., and Baverel, G. (1993). A simple model for alanine metabolism in isolated rat hepatocytes. *Biochem. Biophys. Acta.* **1175:** 161-173.

Max, S.R., and Silberlgeld, E.K. (1987). Skeletal muscle glucocorticoid receptor and glutamine synthetase activity in the wasting syndrome in rats treated with 2, 3, 7, 8 -tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmcol.* **87:** 523-527.

McConnell, E.E. and Moore, J.A. (1979). Toxicopathology characteristics of the halogenated aromatics. *Ann. N.Y. Acad. Sci.* **320:** 138-150.

McGrogan, M., Simonsen, C. C., Smouse, D. T., Farnham, P. J., and Schimke, R. T. (1985). Heterogeneity at the 5' terminal of mouse dihydrofolate reductase mRNA. *J. Biol. Chem.* **260:** 2307-2314.

McKim Jr, J.M., Marien, K., Schaup, H.W., and Selivonchick, D.P. (1991). Alteration of hepatic acetyl CoA carboxylase by . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Lipids.* **26:** 521-525.

Mieskes, G., Kuduz, J., and Soling, H-D. (1987). Are calcium-dependent protein kinase involved in the regulation of glycolytic/gluconeogenic enzymes? *Eur. J. Biochem.* **167:** 383-389.

Mocarelli, P., Marocchi, A., Brambilla, P., Gerthoux, P.M., Colombo, L., Mondonico, A., and Meazza, L. (1991). Effects of dioxin exposure in humans at Seveso, Italy. *Banbury Report* **35:** 95-100.

Mollering, H. (1985). In Methods of Enzymatic Analysis, 3 rd., Bergmeyer. H., and BraB1, M., eds., pp 2-12, VCH, Germany

Moore, R.W., Parsons, J.A., Bookstaff, R.C. and Peterson, R.E. (1985). Androgenic deficiency in male rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* **79:** 99-111.

Morgan, J. E. and Whitlock, J. P. Jr. (1992). Transcription-dependent and transcription-independent nucleosome disruption induced by dioxin. *Proc. Natl. Acad. Sci. USA* **89:** 11622-11626.

Nambu, J. R., Lewis, J. O., Wharton, K. A. Jr., and Crews, S. T. (1991). The drosophilar single-minded gene encodes a helix-loop-helix protein that act as a master regulator of CNS midline development. *Cell* **20:** 1157-1167

Nebert, D.W. (1989). The Ah locus: Genetic difference in toxicity cancer, mutation, and birth defects. *Crit. Rev. Toxicol.* **20:** 137-152.

Nebert, D.W., and Gonzalez, F.J. (1987). P450 genes: structure, evolution, and regulation. *Annu. Rev. Biochem.* **56:** 945-993.

Ochman, H., Medhora, M. M., Garza, D., and Hartl, D. L. (1990). Amplication of flanking sequences by inverse PCR. *In PCR protocols: a guide to methods and amplications*, Innis, M. A., Gelfanf, D. H., Sninsky, J. J., and White, T. J. eds., Acdemic Press: 219-227.

Okey, A.G., Vella, L.M. and Harper, P.A. (1989). Detection and characterization of a low affinity form of cytosolic receptor in livers of mice nonresponsive to induction of cytochrome P450 by 3-methylchloanthrene. *Mol. Pharmacol.* **35:** 823-830.

Olson, J. L., Holscher, M. A., and Neal, R. A. (1980). Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the Golden Syrian Hamster. *Toxicol. Appl. Pharmacol.* **55:** 67-78.

Owens, I.S. (1977). Genetic regulation of UDP-glucuronosyltransferase induction by polycyclic aromatic compounds in mice. Co-segregation with arylhydrocarbon(benzo{a}pyrene) hydroxylase induction. *J. Biol. Chem.* **252:** 2287-2833.

Pace, W. R., Olsen, G. J., and Woses, C. R. (1988). Ribosomal RNA phylogeny and the primary line of evolutionary descent. *Cell.* **45:** 352-326.

Palecek, E. (1991). Local supercoil-stabilized DNA structures. *Critical. Rev. Biochem. Mol. Biol.* **26:** 151-226.

Panyutin, I. G. and Wells, R. D. (1991). Nodule DNA in the $(GA)_{37} \bullet (CT)_{37}$ insert in superhelical plasmids. *J. Biol. Chem.* **267:** 5495-5501.

Perdew, G. (1990). Chemical cross-linking of the cytosolic and nuclear forms of the Ah receptor in hepatoma cell line 1c1c7. *Biochem. Biophys. Res. commun.* **182:** 55-62.

Perdew, G. H. (1988). Association of the Ah receptor with the 90-kDa heat shock protein. J. Biol. Chem. 263: 13802-13805.

Peterson, R.E., Seefeld, M.D., Christian, B.J., Potter, C.L., Kellin, C.K., and Keesey, R.E. (1984). The wasting syndrome in 2, 3, 7, 8 -tetrachlorodibenzo-p-dioxin toxicity: Basic features and their interpretation. *Banbury Report* **18:** 291-308.

Pirkle, J. L., Wolfe, W. F., and Patterson, D. G. (1989) Estimate of half-life of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Vietnam veterans of Operation Ranch Hand. *J. Toxicol. Environ. Health* **27:** 165-171.

Pitot, H.C., Goldsworthy, T., Campbell, H.A., and Poland, A. (1980). Quantitative evaluation of the promotion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxins of hepatocarcinogenesis from dietylnitrosamine. *Cancer Res.* **40:** 3616-3620.

Poland, A. , Teitelbaum, P., Glover, E., and Kende, A. (1989). Stimulation of in vivo hepatic uptake and in vitro hepatic binding of [125I]2-Iodo-3,7,8-trichlorodibenzo-p-dioxin by the administration of agonists for the Ah receptor. *Mol. Pharmacol.* **36:** 121-127.

Poland, A. and Glover, E. (1980). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Segregation of toxicity with the Ah locus. *Mol. Pharmacol.* **17:** 86-94.

Poland, A. and Knutson, J.C. (1982). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanisms of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22:** 517-514.

Poland, A., Teitelbaum, P., Glover, E., and Kende, A. (1989). Stimulation of *in vivo* hepatic uptake and in vitro binding of [^{125}I]2-Iodo-3,7,8-tetrachlorodibenzo-*p*-dioxin by the administration of agonists for the Ah receptor. *Mol. Pharmacol.* **36:** 121-127.

Poland, A.P. and Glover, E. (1976). Stereospecific, high affinity binding of . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. *J. Biol. Chem.* **251:** 4936-4946.

Poland, A.P., Glover, E., Robinson, J.R., and Nebert, D.W. (1974). Genetic expression of arylhydrocarbon hydroxylase activity. Induction of monooxygenase activities and cytochrome P450 formation by . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mice genetically "nonresponsive" to other aromatic hydrocarbons. *J. Biol. Chem.* **249:** 5599-5606.

Pollenz, R. S. (1996). The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Pharmacol. Exp. Therapeutics* **49:** 391-398.

Puga, A., and Nebert, D. W., and Carrier, F. (1992). Dioxin induces expression of c-fos and c-jun proto-oncogenes and a large increase in transcription factor AP-1. *DNA Cell Biol.* **11:** 269-281.

Putney, J. W. and Bird, G. S. L. (1993). The inositol phosphate-calcium signaling system in non-excitable cells. *Endor. Rev.* **14:** 610-631.

Qin Lu, Wallrath, L. L., Granok, H., and Elgin. S. C. R. (1993). $(CT)_n$:$(GA)_n$ repeats and heat shock elements have distinct roles in chromatin structure and transcritpional activation of the *Drosophila hsp26* gene. *Mol. Cell. Biol.* **13:** 2802-2814.

Rando, R. F., DePaolis, L., Durland, R. H., Jayaraman, K., Kessler, D. J., and Hogan M. E. (1994). Inhibition of T7 and T3 RNA polymerase directed transcription elongation *in vitro. Nucleic Acid Res.* **22:** 678-685.

Reyes, H., Reisz-Porszasz, S., and Hankinson, O. (1992). Identification of Ah receptor nuclear translator protein (ARNT) as a component of the DNA binding form of the Ah receptor. *Sciences* **256:** 1193-1195.

Robinson, B.H., Oei, J., Sherwood, W.G., Applegarth, D., Wong, L. Haworth, J., Goodyer,P. Casey, R. and Zaleski, L.A. (1984). The molecular basis for the two different clinical presentations of classical pyruvate carboxylase deficiency. *Am. J. Human Gen.* **36:** 283-252.

Roeder, R. G. (1996). The role of general initiation factors in transcription by RNA polymerase II. *Trends in Biochem. Sci.* **21:** 327-335

Roy, A. L., Meisteremst, M., Pognonec, P., and Roeder. (1991). Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. *Nature* **354:** 245-248.

Rushmore, T.H. and Pickett, C.B. (1990). Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene: characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J. Biol. Chem.* **265:** 14648-14653.

Rutledge, S.L., Snead, O.C., Kelly, D.R., Kerr, D.S., Swann, J.W., Spink, D.L., and Martin, D.L. (1989). Pyruvate carboxylase deficiency: Acute exacerbation after ACTH treatment of infantile spasm. *Pediatr. Neurol.* **5:** 249-252.

Ryu, B. -W., Shukla, R., Sparrow, B. R. Selivonchick, D. P., and Schaup, H. W. (1995). Ah receptor involvement in mediation of pyruvate carboxylase levels and activity in mice given 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biochem. Toxicol.* **10:** 103-109.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds; Environmental

and mechanistic considerations which support the development the toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* **21:** 219-238.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Identification of bacterial colonies that contain recombimant plasmids. *In Molecular Biology, a laboratory manual, 2nd edition,* Cold Spring Harbor Laboratory Press: 1.85-1.86.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Strategies for cloning in plasmid vectors. *In Molecular Biology, a laboratory manual, 2nd edition,* Cold Spring Harbor Laboratory Press: 1.53-1.84.

Scheuplein, R. J., Gallo, M. A., and van der Heijden, D. A. (1991). Epilogue, *Banbury Report* **35:** 489-490.

Schwertz, B. A., Norris, J. M., Sparschu G. L., Rowe, V. K., Gehring, P. J., Emerson, J. L., and Gerbig, C. G. (1973). Toxicology of chlorinated dibenzo-p-dioxins. *Environ. Health Perspect.* **5:** 87-99.

Sgaramella, V. and Khorana, H. G. (1972). Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymatic joining of the chemically synthesized polynucleotide to form the DNA duplex representing nucleotide sequence 1 to 20. *J. Mol. Biol.* **72:** 427-444.

Shih, H.-M. and Towle, H. C. (1992). Definition of the carbohydrate response element of the rat S14 gene. *J. Biol. Chem.* **267:** 13222-13228.

Silkworth, J.B., Cutler, D.S. and Sack, G. 91989). Teratology of . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a complex environmental mixture from the Love Canl. *Fundam. Appl. Toxicol.* **13:** 1-15.

Singleton, C. K., Klysik, J., Stirdivant, S. M., and Wells, R. D. (1982). Left-handed Z-DNA is induced by supercoiling in physiologic ionic condition. Nature 299: 312-316.

Smale, S. T. and Baltimore, D. (1989). The "Initiator" as a transcription control element. *Cell* **57:** 103-113.

Sparrow, B.R., Thompson, C.S., Ryu, B.-W, Selivonchick, D.P. and Schaup, H.W., 1994. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced alterations of pyruvate carboxylase levels and lactate dehydrogenase isozyme shifts in C57BL/6J male mice. *J. Biochem. Toxicol.* **9:** 329-335.

Staddon, J. M. and Hansford, R. G. (1989). Evidence indicating that the glucagon-induced increases in cytoplasmic free Ca^{2+} concentration in

hepatocytes is mediated by increases in cyclic-AMP concentration. *Eur. J. Biochem.* **179:** 47-52.

Stahl, B.U., Beer, D.G., Weber, L.W.D. and Rozman, K. (1993). Reduction of hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is due to decreased mRNA levels. *Toxicology.* **79:** 81-95.

Strobel, S. A. and Dervan, P. B. (1990). Site-specific cleavage of a yeast chromosome by oligonucleotide-directed triple-helical formation. *Science* **249:** 73-75.

Takahashi, J. S. (1992). Circadian clock genes are ticking. *Science* **258:** 238-240.

Takimoto, R., Lindahl, R. and Pitot, H.C. (1991). Superinduction of 2,3,7,8-tetrachlordibenzo-*p*-dioxin-inducible expression of aldehyde dehydrogenase by the inhibition of protein synthesis. *Biochem. Biophys. Res. Commun.* **180:** 953-959.

Takimoto, R., Lindahl, R., and Pitot, H.C. (1991) Superinduction of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin- inducible expression of aldehyde dehydrogenase by the inhibition of protein synthesis. *Biochem. Biophys. Res. Commun.* **180:** 953-959.

Travers, A. A. (1992). The reprogramming of transcriptional competence. *Cell* **69:** 573-575.

Turteltaub, K.W., Felton, J.S., Gledhill, B.L., Vogel, J.S., Southon, J.R., Caffee, M.W., Finkel, R.C., Nelson, D.E., Procter, I.D., and David, J.C. (1990). Accelerator mass spectrometry in biomedical dosimetry: Relationship between low-level exposure and covalent binding of heterocyclic amine carcinogens to DNA. *Proc. Natl. Acad. Sci. USA* **87:** 5288-5292.

Umbreit, T.H., Hesse, E.J., Macdonald, G.J. and Gallo, M.A. (1987). Effects of TCDD-estradiol interactions in three strains of mice. *Toxicol. Lett.* **40:** 1-9.

Valera. A., Pujol, A., Gregori, X., Riu, E., Visa, J., and Bosh F. (1995). Evidence from transgenic mice that myc regulates hepatic glycolysis. *FASEB* **9:** 1067-1078.

Vecchi, A., Sironi, M., Canegreti, M. A., Recchis, M., and Garattini, S. (1983). Immunosuppressive effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in strain

of mice with different susceptible to induction aryl hydrocarbon hydroxylase. *Toxicol. Appl. Pharmacol.* **68:** 434-441.

Vos, J.G., and Moore, J.A. (1974). Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxins. Int. Arch. *Allergy Appl. Immunol.* **47:** 777-791.

Vos, J.G., Moore, J.A. and Zinkl, J.G. (1973). Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system of laboratory animals. *Environ. Health Perspect.* **5:** 149-162.

Vos, J.G., Van Loveren, H. and Schuurman, H.-J. (1991). Immunotoxicology of dioxin: Immune function and host resistance in laboratory animals and humans. *Banbury Report* **35:** 79-93.

Wassom, J.S., Huff, J.E. and Lortriano, N.A. (1977). A review of the genetic toxicology of chlorinated dibenzo-p-dioxins. *Mutat. Res.* **47:** 141-160.

Weber, L.W.D., Greim, H., and Rozman, K. (1987). Metabolism and distribution of [^{14}C] glucose in rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *J. Toxicol. Environ. Health.* **22:** 195-206.

Weber, L.W.D., Lebofsky, M., Greim, H., and Rozman, K. (1991). Key enzymes of gluconeogenesis are dose-dependently reduced in . 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Arch. Toxicol.* **65:** 119-123.

Weber, L.W.D., Lebofsky, M., Stahl, B.U., Gorski, J.R., Muzi, G., and Rozman, K. (1991). Reduced activities of key enzymes of gluconeogenesis as possible cause of acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats. *Toxicology.* **66:** 133-144.

Weber, L.W.D., Lebofsky, M., Stahl, B.U., Kettrup, A., and Rozman, K. (1992). Comparative toxicity of four chlorinated dibenzo-*p*-dioxins (CDDs) and their mixture. Part II: Structure-activity relationship with inhibition of hepatic phosphoenolpyruvate carboxykinase, pyruvate carboxylase, and r-glutamyl transpeptidase activities. *Arch. Toxicol.* **66:** 478-483.

Weiberg, M.B., and Utter, M.F. (1979). Effect of thyroid hormone on the turnover of rat live pyruvate carboxylase and pyruvate dehydrogenase. *J. Biol. Chem.* **254:** 9492-9499.

Whitelaw, M. L., Gustafsson, J. A., and Poellinger, L. (1994). Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor Arnt: inducible versus constitutive modes of regulation. Mol. Cell. Biol. 14: 8343-8355.

Whitlock, Jr, J.P., (1990). Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* **30:** 251-277.

Whitlock, Jr, J.P., (1993). Mechanistic aspects of dioxin. *Chem. Res. Toxicol.* **6:** 754-763.

Williamson, J. R. and Monck, J. R. (1989). Hormone effects on cellular Ca^{2+} fluxes. *Annu. Rev. Physiol.* **51:** 107-124

Yang, E.K., Radominska, A, Winder, B.S.,and Dannenberg, A.J. (1993). Dietary lipids coinduce xenobiotic metabolizing enzymes in rat liver. *Biochem. Biophys. Acta.* **1168:** 52-58.

Zhang, J., Xia, W. -L., Brew, K., and Ahmad, F. (1993). Adipose pryuvate carboxylase; Amino acid sequence and domain structure deduced cDNA sequencing. *Proc. Natl. Acad. Sci. USA.* **90:** 1766-1770.

Zinkl, J.G., Vos, J.G., Moore, J.A., and Gupta, B.N. (1973). Hematological and clinical chemistry effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals. *Environ. Health Perspect.* **5:** 111-118.