

TRANSCRIPTIONAL REGULATION OF RAT PEROXISOMAL
ENOYL-CoA HYDRATASE/3-HYDROXYACYL-CoA DEHYDROGENASE

By

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ABSTRACT

Structurally diverse peroxisome proliferators induce in rodents a remarkable increase in the activities of peroxisomal fatty acid β -oxidation enzymes. The transcriptional induction of these peroxisomal enzymes in liver has been mechanistically associated with the subsequent pleiotropic responses such as peroxisome proliferation, hypolipidemia, hepatomegaly, and hepatocellular carcinomas. This project was designed to investigate the mechanisms of transcriptional regulation of the rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) by hypolipidemic peroxisome proliferators.

In search of a peroxisome proliferator responsive element in the promoter region of the HD gene, the 5.8 kbp upstream region of the HD gene and its deletion mutated fragments were subcloned into a reporter plasmid that contained a luciferase gene. The recombinant plasmids were introduced into H4IIEC3 cells in the presence of ciprofibrate, a hypolipidemic drug. Luciferase activity was measured to assay the inducibility of the promoter mutants. The sequence between nucleotides -3040 and -2845 behaved as a minimal peroxisome proliferator responsive element (PPRE). DNase I footprint analysis of this PPRE fragment with H4IIEC3 nuclear proteins revealed two protected regions. Region I contains consensus hormone responsive motifs: two inverted TGACCT-like

sequences followed by a third. Region II contains two protein binding sites: a site containing several CACCC boxes and the other consisting of three directly repeated TGACCT core motifs. In transient transfection assays, the oligonucleotide containing these TGACCT direct repeats, nucleotides -2956 to -2919, conferred a ciprofibrate response, which was independent of its position and orientation relative to the transcription start site. In mobility shift assays, this sequence was recognized by liver specific nuclear proteins.

The three TGACCT-like motifs were further characterized by mutational analysis. Mutations that reduced the intensity of the liver factor binding in mobility shift assays resulted in a decrease in the responsiveness to ciprofibrate in transfection assays. Mutation of a single G residue in the middle core motif completely abolished both the factor binding *in vitro* and the responsiveness to ciprofibrate *in vivo*. Modulation of this core HD PPPE involved several known transcription factors. PPAR, RXR α , COUP-TF1, and, weakly, HNF4, were detected to be present in the complex of liver factors with HD PPPE by mobility shift assays with the addition of the respective antibodies. This result was confirmed by similar experiments with the *in vitro* translated products of mRNAs of these proteins. PPAR and RXR α required the second and third repeats of the HD PPPE whereas COUP-TF1 and, probably also HNF4, recognized the first and second repeats. PPAR and RXR α cooperatively transactivated a reporter gene via this HD

PPRE in response to ciprofibrate in several cell lines. COUP-TF1 antagonized the up regulation by PPAR and RXR α .

In conclusion, the major *cis* element responsible for the ciprofibrate-induction of the HD gene consists of three directly-repeated TGACCT-like motifs. Its function is independent of its position and orientation relative to the transcription start site. Binding of liver specific factors to the element is a prerequisite for its ciprofibrate responsiveness. The overall integrity of the three motifs and the correct spacing between the motifs are critical determinants for the nuclear factor recognition and the transcriptional activation. The transcriptional regulation of the HD gene is achieved by a cooperation of several liver specific transcription factors.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
ACBP	acyl-CoA binding protein
AOx	fatty acyl-CoA oxidase
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	DNA complementary to RNA
Ci	Curie(s)
CoA	coenzyme A
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
CPS	carbamoyl phosphate synthetase
CRABP	cellular retinoic acid binding protein
CYP	cytochrome P450
cpm	counts per minute
Da	dalton
DEHP	2-di-(2-ethylhexyl)phthalate
DMS	dimethyl sulfate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside 5'-triphosphate
DDT	dithiothreitol
DR _x	direct repeat of TGACCT motifs with x nucleotides in between
EDTA	ethylenediaminetetraacetic acid
EHXA	2-ethylhexanoic acid
EHXO	2-ethylhexanol
ER	endoplasmic reticulum
ER	estrogen receptor
FABP	fatty acid binding protein
GR	glucocorticoid receptor
g	gram(s)

GTP	guanosine 5'-triphosphate
HD	enoyl-CoA hydratase/3-hydroxy acyl-CoA dehydrogenase
HEPES	n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HNF	hepatocyte nuclear factor
HRE	hormone responsive element
HSP	heat shock protein
IgG	immunoglobulin G
k	kilo
luc	luciferase gene
μ	micro
m	milli
M	molar
mA	milli Ampere
min	minute(s)
mol	mole(s)
mRNA	messenger ribonucleic acid
n	nano
NP-40	Nonidet P-40
NTP	nucleoside 5'-triphosphate
Oct-1	octamer nucleotide binding protein
OD _x	optical density measure at the wavelength of x nanometers
p	pico
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMP	peroxisome membrane protein
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator responsive element
RA	retinoic acid
RAR	retinoic acid receptor
RXR	9-cis retinoic acid receptor
RNA	ribonucleic acid
rpm	revolutions per minute

SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate buffer
TBE	tris-buffered saline plus Tween 20
TE	10 mM Tris-HCl (pH 7.5), 1mM EDTA
Tris	Tris(hydroxymethyl)aminoethane
TR	thyroid hormone receptor
tRNA	transfer ribonucleic acid
UDP	uridine diphosphate

INTRODUCTION

Although the discovery of the physical form of DNA and the deciphering of the genetic code are revolutionary landmarks of modern life science, a complete description of the DNA sequence of a genome may provide little understanding of the organism. Gene control, on the other hand, represents a central issue. It is the three parameters of gene control: the concentration of a specific mRNA, the frequency with which this mRNA is translated, and the stability of the protein that are responsible for the specific characteristics of each cell at a given time (Young, 1991; Hershey, 1991).

The transcriptional induction of peroxisomal genes in response to a variety of chemicals is one of the most interesting models to study the mechanisms of transcriptional regulation of inducible genes. Chemically induced overexpression of the peroxisomal genes often provokes profound changes in fatty acid metabolism, heat overproduction, peroxisome proliferation, hepatomegaly and hepatic carcinogenesis (Reddy and Lalwani, 1988). Many peroxisome proliferators are clinically or industrially important, for example: hypolipidemic drugs and plasticizers (Goldfischer and Reddy, 1984; Lock et al., 1989; Van den Bosch et al., 1992). Studies of these chemicals have in the past provided insight into the structure and functions of peroxisomes, including the

identification of a fatty acid β -oxidation system in the organelle. Recent studies of the upstream regions of two peroxisomal β -oxidation enzymes, fatty acyl-CoA oxidase (AOx) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), have led to the discovery of new transcription factors, such as the peroxisome proliferator activated receptors (PPARs), and new *cis*-acting elements, such as the peroxisome proliferator responsive elements (PPREs) (Green, 1992; Green et al., 1992).

The focus of this thesis is on a PPRE in the HD promoter region and its interaction with transcription factors that are activated upon treatment of peroxisome proliferators. The following review will be a brief introduction to the current knowledge on peroxisomal β -oxidation enzymes, their induction by peroxisome proliferators, the biological consequences and the possible mechanisms of peroxisome proliferation.

1.1 Peroxisomes

1.1.1 General Characteristics

Peroxisomes are ubiquitous cytoplasmic organelles present in not only plant and animal cells but also in protozoa and fungi (Goldfischer and Reddy, 1984). Typical mammalian peroxisomes are single membrane-limited organelles with some variance in shape and size, measuring 0.1 μm in brain cells or fibroblasts and 0.5-1.5 μm in liver or kidney cells. Delimited by the membrane is a

homogeneous matrix of moderate electron density. Hepatic peroxisomes in different species contain distinctly different crystalloid cores, nucleoids, which are believed to be associated with urate oxidase (Goldfischer and Reddy, 1984). Human peroxisomes are often larger than 0.5 μm in diameter. They are anucleoid without detectable urate oxidase activity but often contain marginal plates, another common type of crystalline inclusion (Sternlieb and Quintana, 1977). Similar plates in rat liver peroxisomes mainly contain a B-isoenzyme of L- α -hydroxyacid oxidase (Mannaerts and Veldhoven, 1993).

It is now well accepted that peroxisomes arise from growth and division of preexisting peroxisomes (Fahimi et al., 1993). Peroxisomes contain no nucleic acids. All the peroxisomal proteins are encoded in the nucleus and are synthesized on free ribosomes (Rachubinski et al., 1984; Fujiki et al., 1984; Suzuki et al., 1987; Subramani, 1992). Most of them are synthesized in their final size without a cleavable topogenic peptide and are imported post-translationally into peroxisomes (Subramani, 1992; Hoop and Geert, 1992).

Absence of peroxisomes could be lethal as is seen in Zellweger's syndrome, a human autosomal recessive disease (Moser et al, 1990). The patients suffer from a malfunction of virtually every organ and hardly survive beyond the fourth month. Its major characteristic is the absence of functional peroxisomes, which

leads to the failure of peroxisomal β -oxidation and perhaps also plasmalogen synthesis (Aikawa et al., 1991; Santos et al., 1988 and 1992; Suzuki et al., 1989). Biosynthesis of peroxisomal enzymes still seems normal, only they are mislocated to the cytosol. In hepatocytes, empty "peroxisomal ghosts" have been found, which retain a membrane structure with membrane proteins of 150, 70, 35, and 22 kDa (Tager et al., 1990; Wilson, 1991; Suzuki et al., 1989). Recent studies have revealed that certain impaired peroxisomal membrane proteins are responsible for the failure of protein targeting to peroxisomes (Hashimoto et al., 1986; Shimozawa et al., 1992; Thieringer and Raetz, 1993). Thus, although almost all of the peroxisomal proteins have their counterparts in some other subcellular structures (Small et al., 1990), the biochemical functions of peroxisomes are not replaceable.

1.1.2 *Biochemical Functions of Peroxisomes*

Peroxisomes house more than 50 enzymes (Small et al., 1990). These include one or more flavin oxidases that generate H_2O_2 , catalase that catalyzes the decomposition of H_2O_2 into O_2 and H_2O , and H_2O_2 utilizing enzymes that oxidize various compounds by H_2O_2 . With these enzymes, peroxisomes are active in β -oxidation of fatty acids and fatty acid derivatives such as β -oxidation of the side chain of cholesterol to form bile acids; plasmalogen biosynthesis; cholesterol metabolism; the catabolism of purine, amino acid, and polyamine; glyoxylate utilization; and the inactivation of reactive oxygen species such as H_2O_2 , superoxide anions, and epoxides

(Subramani, 1992). Lately, some unique metabolic pathways have been found in the organelle, for example, the β -oxidation from the ω -end of leukotriene, a potent mediator in inflammatory processes and hypersensitivity reactions (Jedlitschky et al., 1991).

More than half of the peroxisomal proteins are involved in lipid metabolism. A peroxisomal β -oxidation system carries on stepwise β -oxidation of fatty acids principally like the β -oxidation in mitochondria (Mannaets and Veldhoven, 1992; Small et al., 1990). Peroxisomal β -oxidation, however, prefers those substrates with very long chains such as tetracosanoic (C24:0) and hexacosanoic (C26:0) acid. This is thought to be due to the fact that the peroxisomal acyl-CoA synthetase, an integral membrane protein, has activity only towards very-long-chain fatty acids, unlike the mitochondrial acyl-CoA synthetase which prefers the medium- and short-chain fatty acids. Different from mitochondria, peroxisomal β -oxidation is cyanide-insensitive and carnitine-independent. It does not couple the oxidation to phosphorylation and does not degrade fatty acids completely (Van den Bosch et al., 1992). The products are shortened acyl-CoAs which are converted by a peroxisomal carnitine acyl-transferase, a soluble matrix protein, to carnitine esters for further oxidation in mitochondria (Small et al., 1990).

Peroxisomal and mitochondrial β -oxidation enzymes are products of different genes. In rats, for instance, the peroxisomal acyl-CoA

oxidase (AOx) is the product of a single gene (Miyazawa et al., 1987; Schepers et al., 1990). Alternative splicing results in two different mRNAs (Bell and Elcombe, 1991). Three polypeptides with relative molecular masses of 72, 52, and 22.5 kDa are present in the purified preparation of the enzyme. The two smaller ones are probably the products from post-translational proteolysis of the 72 kDa species. It is believed that at least two oxidases are formed from these peptides, a 427 kDa and a 145 kDa protein (Miyazawa et al., 1987; Schepers et al., 1990). The exact subunit composition and biochemical functions of the different AOx species remain unclear.

The second and third reactions of β -oxidation in peroxisomes are catalyzed by a monomeric bifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), while the same task in mitochondria is carried out by two separate proteins (Small et al., 1990). The peroxisomal HD is a 77 kDa protein encoded by a single copy gene (Ishii et al., 1987). There is no evidence for the presence of more than one mRNA or protein. By comparison of the amino acid sequences of this enzyme and the three corresponding enzymes in mitochondria, it has been proposed that the peroxisomal HD has both isomerization and hydration activities in its amino-terminal domain, while its dehydrogenase activity is in the carboxyl-terminal domain (Palosaari and Hiltunen, 1990; Palosaari et al., 1991). The specific isomerase activity is, however, only 1% of that of a separate peroxisomal isomerase of 30 kDa (Yokota et

al., 1989; Tomioka et al., 1991), and it is not detected in a report by a different author (Ishii et al., 1987). In this review, therefore, the name HD is used for the dominant bifunction of the enzyme. Finally, 3-ketoacyl-CoA thiolase that catalyzes the last reaction of the peroxisomal β -oxidation cycle has two isoforms encoded by two different genes (Bodnar and Rachubinski, 1990; Hijikata et al., 1987; Hijikata et al., 1990).

More importantly, peroxisomes contain catalase, the marker enzyme of the organelle which is not present in mitochondria or other subcellular structures (Korneluk et al., 1984; Thieringer et al., 1991). Catalase is the product of a single gene, a tetrameric heme-protein consisting of four identical 60 kDa units (Furuta et al., 1986; Quan et al., 1987). Distinct from mitochondria, the peroxisomal AOX transfers the electrons from its dehydrogenation of fatty acyl-CoA directly to O_2 to form H_2O_2 which is handed over to catalase for further degradation. This unique H_2O_2 -mediated branch renders peroxisomes with profound roles in many biological and pathological processes (Van den Bosch et al., 1992; Osumi, 1993).

1.2 Biological Effects of Peroxisome Proliferators

1.2.1 Peroxisome Proliferators

Peroxisome proliferators are a group of structurally diverse chemicals (Reddy and Lalwani, 1988). The major categories are fibrate hypolipidemic drugs, phthalate plasticizers, certain

polychlorinated biphenyl isomers, chlorophenoxy acid herbicides, certainazole antifungal drugs, perfluorinated fatty acids, high-fat diets, vitamin E, retinoids, and dehydroepiandrosterone (Sakuma et al., 1992, Song et al., 1989).

Fibrates are a class of aryloxyacetic acid derivatives that are developed to effectively reduce total plasma triglycerides and cholesterol and have been used to combat cardiovascular diseases (Reddy and Lalwani, 1988; Catapano, 1992). Examples include benzafibrate, fenofibrate, gemfibrozil, and two extensively studied drugs: clofibrate (p-chlorophenoxyisobutyric acid) and ciprofibrate (2-[4-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropionic acid). Twice daily, 700 mg of clofibrate or other fibric acid reduced total plasma triglycerides and total plasma cholesterol (Coronary Drug Project Group, 1975). A reduction in the incidence of cardiac events, especially the non-fatal myocardial infarction, has been repeatedly confirmed. Community-scale preventive usage of these drugs is not recommended due to the observed excess of cause-unknown death among people who took the drug for a long period, (Coronary Drug Project Group, 1975). The local and short-term usage, however, still benefits patients with coronary diseases. In some clinical situations, such as after heart transplantation, the fibrate drugs are critical for post-operation survival (Peters et al., 1993). It is the goal of the pharmaceutical industry to develop efficient new drugs with little side effects (Todd and Ward, 1988; Lalwani et al., 1985).

Phthalate plasticizers are widely used in the modern plastic industry in the formulation of a variety of plastics and elastomeric materials (Reddy and Lalwani, 1988; Thomas and Thomas, 1984). Medical applications include heart valves, vascular grafting materials, intrauterine devices, catheters, dialyzing units, blood transfusion sets and disposable syringes. The name phthalate designates esters of phthalic acid with straight- or branch-chain alcohols. 2-Di-(2-ethylhexyl)phthalate (DEHP) is the most common one in this category. It is extremely insoluble in water but soluble in blood and in other lipoprotein containing materials. Through leaching, diffusion or other sorptive processes, DEHP migrates into transfusion fluids and thus has the potential of causing toxic reactions. Phthalate esters have also been found to migrate into milk, beef, chicken breast and other high-fat foods. Deep-frying fat contains traceable amounts of DEHP. The detection of DEHP in human tissues especially those from people without a history of blood transfusion indicates that human exposure may not be accidental. There even exists the possibility of endogenous biosynthesis of these chemicals by man or his associated microflora (Thomas and Thomas, 1984).

In contrast to their heterogeneous chemical structures, peroxisome proliferators have similar multiple biological effects, including a decrease in the serum level of triglycerides and cholesterol, a selective induction of several peroxisomal and non-peroxisomal enzymes, peroxisome proliferation, hepatomegaly, and

hepatocarcinomas (Reddy and Rao, 1992).

1.2.2 Peroxisome Proliferation

The most remarkable feature of peroxisomes is their inducibility. A drastic increase in the size and number of the organelle occurs in response to xenobiotic compounds. The appearance of the induced peroxisomes is largely the same as the preexisting ones (Fahimi et al., 1993). Some peroxisome-forming sheets (peroxisomal reticulum) distinct from the endoplasmic reticulum have been observed. It is a structure interconnecting several peroxisomes, which has not been seen during normal cell growth and division (Fahimi et al., 1993).

All peroxisome proliferators are inducers of peroxisomal β -oxidation enzymes (Osumi et al., 1990; Malki et al., 1990; Lazarow and de Duve, 1976; Van den Bosch et al., 1992). In rodents, up to a 30-fold increase in peroxisomal fatty acid β -oxidation proteins is often reported (Chen and Crane, 1992; Chen et al., 1988; Hartl and Just, 1987). Also occurring is a co-induction of several metabolically related enzymes such as long-chain acyl-CoA synthetase (Suzuki et al., 1990; Lazo et al., 1991) and certain peroxisomal integral membrane polypeptides (Crane, et al., 1987; Hartl & Just, 1987; Bodnar & Rachubinski, 1991).

The induction of both peroxisomal β -oxidation enzymes and integral membrane proteins is at the transcription level (Reddy et al., 1986; Hartl and Just, 1987; Hijikata et al., 1990; Bodnar and

Rachubinski, 1990 and 1991; Thangada et al., 1989; Chen and Crane, 1992;). For peroxisomal AOX, the ratio of the two types of transcripts is drastically raised during peroxisome proliferation, possibly due to the regulation of differential splicing (Bell and Elcombe, 1991). Transcriptional regulation is, however, the event clearly relevant to peroxisome proliferation. It is the magnitude of the increase in the total mRNA of these inducible genes and the subsequent accumulation of the enzymes that correspond reasonably with the magnitude of the increase in the numerical measurements of hepatic peroxisome population (Lock et al., 1989).

Not all the peroxisomal enzymes are induced on the same scale. HD has been repeatedly reported to be the most responsive (Lock et al., 1989; Alvares et al., 1990). The AOX of 427 kDa is noninducible while the 145 kDa isoenzyme is inducible (Miyazawa et al., 1987; Schepers et al., 1990). Thiolase 1, which is normally undetectable, becomes abundant upon induction, whereas the constitutively expressed thiolase 2 is hardly responsive (Bodnar and Rachubinski, 1990; Hijikata et al., 1990). In the liver of rats treated with clofibrate, only a 69 kDa protein out of the six integral membrane polypeptides is significantly induced (Bodnar and Rachubinski, 1991). Similarly, in mouse liver, only a 68 kDa peroxisomal membrane protein is induced (Crane, et al., 1987; Chen and Crane, 1992).

Most importantly, catalase, the enzyme that handles half the

work load in the peroxisomal β -oxidation pathway, is often induced only two fold (Klucis et al., 1991). This dramatic change in the ratio between H_2O_2 -producing and H_2O_2 -reducing enzymes indicates a partial dysfunction of the induced organelle and has been considered as a principal mechanism for the serious outcomes of chemically induced peroxisome proliferation (Small et al., 1990).

1.2.3 Induction of Cytochrome P450IVA

All the peroxisome proliferators known so far possess a hypolipidemic property despite their structural diversity (Coronary Drug Project, 1975). It has been widely accepted that peroxisome proliferation plays the most important role in the occurrence of hypolipidemia (Reddy and Lalwani, 1988). Yet, an extensive network of lipid metabolic enzymes is concomitantly induced. They may all contribute to hypolipidemia by lowering the plasma level of triglycerides and cholesterol (Reddy and Lalwani, 1988). This network includes microsomal lipid metabolic enzymes (Lock et al., 1989), cytosolic fatty acid binding protein (FABP) and acyl-CoA binding protein (ACBP) (Brandes et al., 1990; Kanda et al., 1990; Heuvel et al., 1993), enzymes involved in the bile acid and cholesterol catabolic pathway, and an LDL receptor (Kawashima et al., 1986; Catapano, 1992; Arand et al., 1991).

The induction of microsomal ω -oxidation of fatty acids is as dramatic as that of the peroxisomal β -oxidation system (Kawashima and Kozuka, 1992). Microsomal ω -oxidation is a cytochrome P450-

dependent pathway consisting of many members of the P450IVA family. Among them, cytochrome P452 (also named P450IVA1, CYP 4A1) and P450IVA6 (CYP 4A6) catalyze the ω -hydroxylation of arachidonic, lauric, and palmitic acids for subsequent oxidation into the corresponding dicarboxylic acids. They are induced by the same group of agents and conditions that cause peroxisome proliferation, including the fibrates, the plasticizers, high fat diet, starvation and diabetes (Kozuka et al., 1991; Hardwick et al., 1987; Moody, et al., 1992). The classic P-450 inducers such as phenobarbital compounds do not induce P450IVA proteins nor peroxisomal enzymes (Driven et al., 1992; Sharma et al., 1988a and 1988b). Lock et al. (1989) have proposed a mechanism whereby peroxisome proliferation is an adaptive cellular response to the increased demand of clearing long-chain fatty acids so as to maintain cellular lipid homeostasis.

The enzyme induction in these two hepatic organelles has been shown to be mechanistically interrelated (Gibson et al., 1990; Sharma et al., 1988a). Two laboratories independently demonstrated that the increase in rat liver P450IVA1 preceded the induction of peroxisomal β -oxidation after administration of clofibrate or high-fat diet (Gibson et al., 1990; Kaikaus et al., 1993). The induction of P450IVA1 protein was biphasic. The first peak appeared one hour after an intraperitoneal dose. The second peak was seen 24 hours later and coincided with the monophasic peak of the peroxisomal β -oxidation proteins (Small et al., 1990). The induction was also via

a transcriptional regulation (Bars et al., 1993). The time course of P450IVA1 mRNA accumulation corresponded approximately to the two-phase induction of the cytochrome P450IVA1 protein and its catalytic activity (Kaikaus et al., 1993). Further, pretreatment of rats with a protein synthesis inhibitor, cycloheximide, blocked the clofibrate-dependent induction of peroxisomal AOX mRNA but had only minimal effect on the induction of P450IVA1 mRNA (Kaikaus et al., 1993). An inactivator of P450IVA enzymes, 1-aminobenzotriazole, also blocked the clofibrate-induced peroxisomal proliferation (Sundseth and Waxman, 1992). Thus, the induction of P450IVA proteins may be not only earlier but also obligatory for peroxisome induction (Milton et al., 1990; Gibson, 1992 and 1993).

1.2.4 *Hepatomegaly and Carcinogenesis*

The liver enlargement caused by peroxisome proliferators results from both hypertrophy and hyperplasia (Small et al., 1990). A predominant increase in peroxisomes and a modest increase in smooth ER correlate with the observed hypertrophy. Hyperplasia occurs through an increased rate of DNA synthesis and mitosis and a decreased rate of apoptosis (Rao and Reddy, 1992; Moody et al., 1992). Hepatomegaly is dose-dependent and is maintained for as long as the chemical is administered. Prolonged exposure leads to increased incidence of hepatocarcinoma in rodents (Reddy and Lalwani, 1988). Many peroxisome proliferators have been classified as B2 carcinogens, that is, probable human carcinogens (Nilsson et al., 1991). Yet, thorough investigations of representative members

of these chemicals have not revealed any significant genotoxic action in any mutagenic testing system (Schiestl and Reddy, 1990; Small et al., 1992; Cattley et al., 1990; Nilsson et al., 1991; Butterworth et al., 1984). Because of this consistently negative genotoxicity, peroxisome proliferators are classified as epigenetic carcinogens (Reddy and Lalwani, 1988).

Many scientists believe that peroxisome proliferators do not cause initiation but act through tumor promotion (Schulte-Herman et al., 1983; Elcombe, 1990). By using the liver carcinogen, aflatoxin B₁, followed by a potent peroxisome proliferator, nafenopin, Kraupp-Grasl et al. (1990) clearly demonstrated that nafenopin possessed a tumor-promoting property. It selectively enlarged preneoplastic foci of a basophilic nature, which resembled the adenoma-related foci found in aging rats. By comparing the young and the old rats in response to the nafenopin treatment alone, they showed that nafenopin was also able to promote the preneoplastic basophilic lesions initiated spontaneously during aging (Cattley and Popp, 1989; Kraupp-Grasl et al., 1990; 1993). These observations support the hypothesis that peroxisome proliferation may lead to ultimate tumor formation via overproduction of H₂O₂ (Tomaszewski et al., 1980). The latter could react with oxygen free radicals produced by many metabolic reactions to give rise to highly reactive hydroxy radicals ($\cdot\text{OH}$), which could severely damage biological membranes, proteins, and nucleic acids (Reddy et al., 1986; Conway et al., 1989; Cattley et al., 1991).

In long-term experiments on rats, the hepatic level of H_2O_2 and the leakage of H_2O_2 from peroxisomes into cytosol were, indeed, increased, and the increase persisted throughout the 78 weeks of administration of a peroxisome proliferator (Tamura et al., 1990). Peroxisome induction was sustained throughout the experiment, and tumorigenesis occurred only after prolonged exposure to the chemicals (Ganning et al., 1987). The magnitude of the increase in the number and volume of peroxisomes also reflected the carcinogenic potency of the chemical and the dose administered (Small et al., 1990). These observations are consistent with an oxidative stress mechanism of hepatic carcinogenesis.

Furthermore, free radical-related damage of macromolecules has been observed following treatment with peroxisome proliferators. In cell culture, lipid peroxidation of the peroxisomal membrane is increased after treatment with clofibrate and DEHP as measured by the level of conjugated dienes, the intermediates of lipid peroxidation (Rao and Reddy, 1991; Lake et al., 1987; Goel et al., 1986; Tomaszewski et al., 1990; Río et al., 1992). Lipofuscin accumulation has been characteristically associated with peroxisome proliferators (Reddy et al., 1989; Marsman et al., 1992). Lipofuscin is supposedly composed of peroxidized debris of cellular lipids and proteins and is often formed under oxidation stress such as during the aging process (Huber et al., 1991; Cattley et al., 1991). In rats, exposure to peroxisome proliferators has led to the detection of DNA single-strand breaks (Fahl, et al., 1984) and

increased levels of 8-hydroxydeoxyguanosine and 5-hydroxymethyl-2'deoxyuridine (HMdU) (Srinivasan et al., 1990; Takagi, et al., 1990). Dietary peroxisome proliferators result in a specific reduction of I (indigeous)-compound levels in the DNA of rat liver (Tamura et al., 1991; Li et al., 1991). This is a covalent, adduct-like modification of DNA. Reduction of I-compound levels has been linked to age-dependent and non-genotoxic carcinogen-induced carcinogenesis.

Additionally, prolonged feeding of clofibric acid and DEHP to rats results in the reduction of cellular antioxidation mechanisms by inhibiting enzymes that metabolize H_2O_2 and organic hydroperoxides, such as cytosolic glutathione peroxidase (GSH-Px), glutathione-S-transferase, and a Mn-requiring superoxide dismutase (Mn-SOD), which has recently been found to be another major peroxisomal defense enzyme against H_2O_2 and O_2^- (Tamura et al., 1990; Glauert et al; 1992). Finally, co-administration of a peroxisome proliferator with free radical scavenging chemicals, such as tert-butyl-4-hydroxyanisole (BHA), reduced tumor incidence, further supporting a role of oxidative stress in peroxisome proliferation-related carcinogenesis (Río et al., 1992).

1.3 The Receptor-Mediated Mechanism

1.3.1 Proximate Peroxisome Proliferators

It is from a pharmacological point of view that a cytosolic

receptor for peroxisome proliferators has been conceived to mediate the transcription of a substantial battery of genes and the ensuing peroxisome proliferation (Reddy and Rao, 1986). Certain cytosolic proteins have been reported to be able to bind to peroxisome proliferators (Alvares et al., 1990). Using affinity chromatography, they have isolated a peroxisome proliferator binding protein which shares homologous amino acid sequence with the heat shock protein, HSP70, and cross-reacts with a monoclonal antibody against the conserved region of the HSP70. Whether this protein is a true receptor for peroxisome proliferator has been, however, questioned because the affinity of the hypolipidemic drugs to the cytosolic fractions is as low as their affinity to non-specific albumin (Milton et al., 1988).

On the other hand, any receptor binding studies should take into account that the parent compounds might not be the true ligands for the putative receptors. Most peroxisome proliferators hold an acidic group, or a potential metabolite with a carboxylic acid moiety. They are activated to acyl-CoA esters *in vivo* by a long-chain non-specific acyl-CoA synthetase, which can be detected in mitochondrial, microsomal and peroxisomal fractions (Bronfman et al., 1986; 1992; Aarsland et al., 1990; Aarsland and Berge, 1991). The rate of metabolic activation of fibrates is qualitatively related to peroxisome proliferation. Structurally related compounds have no induction effect, if they are not activated to their respective CoA derivatives (Aarsland and Berge, 1991). These

authors, therefore, proposed that the acyl-CoA esters be the common pharmacological active species.

DEHP has no acidic group. *In vivo*, however, hydrolysis of one ester bond of DEHP by plasma lipases produces mono-(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol (EHXO). EHXO is rapidly oxidized to 2-ethylhexanoic acid (EHA) (Thomas and Thomas, 1984). Studies have shown that EHA is probably the proximate peroxisome proliferator (Hauck et al., 1989). The S(+)-enantiomer of 2-EHA was found to be a more potent peroxisome inducer than the R(-)-antipode (Macherey et al., 1990). Similarly, studies of clofibrate analogues showed that optimal induction of peroxisomal AOX requires S(-)-orientation of the α -carbon of the carboxylic acid side chain (Esbenshade et al., 1990). Interestingly, when the end point was teratogenicity as measured by the production of neural tube defects, it was the (R)-EHA that was more potent (Hauck et al., 1990). These results strongly suggest that these stereochemicals act as ligands for specific cellular receptors (Chine et al., 1991).

In addition, a true proximate ligand for peroxisome proliferation may possess a specific structure that allows it to accumulate in cells (Berge et al., 1989). It has been shown that long chain dicarboxylic acids (C14-18) induce peroxisomal β -oxidation, but not microsomal ω -hydroxylation (Lock et al., 1989). This induction is P450IVA-independent (Kaikus et al., 1993). The

short- and medium-chain dicarboxylic acids, however, do not induce peroxisomes (Intrasuksri and Feller, 1991), indicating that dicarboxylic function itself is not a critical structural requirement for the induction of peroxisomal enzymes. In contrast, the metabolic stable perfluorinated or sulfur-substituted fatty acids are potent peroxisome proliferators, even though the chain length could be as short as C4 (Berge et al., 1989; Asiedu et al., 1990). It is, therefore, concluded that the structural requirements for a peroxisome-inducing compound include a carboxylic group linked to a hydrophobic backbone and an ability to resist mitochondrial fatty acid β -oxidation (Cannon and Eacho, 1991; Intrasuksri and Feller, 1991; Hertz and Bar-Tana, 1988). This definition predicts that structurally diverse compounds may act through a common mechanism.

1.3.2 *Involvement of a Hormonal Signaling Pathway*

Abundant evidence has suggested that some hormone receptors are potential targets for peroxisome proliferators. Dehydroepiandrosterone, a major secretory steroid hormone from human adrenal glands, has recently been characterized as a potent peroxisome proliferator. It is able to cause a remarkable increase in peroxisomal β -oxidation (Yamada et al., 1991; Frenkel et al., 1990), a concomitant increase in microsomal lauric acid ω -hydroxylation (Sakuma et al., 1992), and subsequent hepatomegaly and hepatocarcinogenesis (Rao et al., 1992). Like other typical xenobiotic peroxisome proliferators, a mechanism of transcriptional

regulation is involved (Sørensen et al., 1992).

Under conditions of starvation or chemically-induced diabetes, increased microsomal ω -hydroxylation and peroxisomal β -oxidation of fatty acids has been observed (Thomas et al., 1989; Orellana et al., 1993), presumably via the accumulation of free fatty acids or their metabolites in liver cells. Fatty acids and dexamethasone synergistically up-regulate the peroxisomal genes in a cell culture system, whereas insulin not only completely blocked all the induction by fatty acids and dexamethasone but also inhibited the basal expression of the three peroxisomal β -oxidation genes (Sørensen et al., 1992; 1993), indicating the existence of discrete cis-elements for functionally diverse trans-acting factors.

It has long been observed that the thyroid and its functions are severely altered by hypolipidemic clofibrates and DEHP (Hertz et al., 1991). In thyroid endocrine cells, hypotrophy of the Golgi apparatus, an increase in lysosomes, and a degeneration of mitochondria are observed in animals fed a clofibrate-containing diet, indicating a persistent hyperthyroidism (Ozalp et al., 1986). The hepatomegaly caused by clofibrate treatment is attenuated in thyroidectomized rats compared to normal rats (Thottassery et al., 1991), suggesting a complex interaction between exogenous peroxisome proliferators and thyroid hormone. On the other hand, hyperthyroidism in rats caused by T_3 has led to the induction of liver and kidney peroxisomes (Fringes and Reith, 1982). Upon

treatment of rats with thyroxine, peroxisomal β -oxidation enzymes and a peroxisomal membrane protein, PMP69, were detected at an elevated level, while noninducible PMPs stayed the same (Kramar et al., 1986; Hartl and Just, 1987). These results indicate the possibility that a nuclear site could be targeted by peroxisome-proliferating agents (Hertz et al., 1991).

1.3.3 *Peroxisome Proliferator Activated Receptor (PPAR)*

The presence of a nuclear binding site raises the possibility that the biochemical mediator for peroxisome proliferation may be a member of the nuclear hormone receptor superfamily. At least 25 mammalian genes known so far belong to this family (Lucas and Granner, 1992). Members of the family include receptors for thyroid hormone, adrenal steroids such as glucocorticoid, vitamin D₃, and retinoic acids, simplified as TR, GR, VD₃R, and RAR, respectively. Binding of a hormonal ligand to its receptor enables the latter to bind to a hormone responsive element (HRE) located upstream of the target genes and to activate transcription of the genes.

Such a rationale prompted Issemann and Green (1990) to screen a mouse liver cDNA library using a probe derived from the conserved sequences of several receptors of this superfamily. One of the clones obtained was named the peroxisome proliferator activated receptor (mouse PPAR or mPPAR). Dimeric fusion receptors made up of the putative ligand binding domain of this PPAR and the DNA binding domain of either oestrogen- or glucocorticoid-receptor was able to

activate oestrogen or glucocorticoid responsive genes in response to peroxisome proliferators of diverse structures. The highest expression of this PPAR is in the liver, the most pronounced target organ of peroxisome proliferators, suggesting that this PPAR may mediate the biological effects of peroxisome proliferators. Amino acid sequence derived from the cDNA reveals that this mPPAR belongs to the superfamily of nuclear hormone receptors. While it shares homology with many members of the superfamily such as hRAR α , hTR β , hVD $_3$ R, and the *Drosophila* ecdysone-inducible receptor homologue E75, the highest similarity is found between this mPPAR and human erb-A. Its putative DNA-binding domain (amino acids 102-166) and ligand-binding domain (amino acids 281-468) are 64% and 38% identical with the corresponding domains of human erb-A, respectively.

Meanwhile, the search for a specific DNA sequence motif responsive to peroxisome proliferators has been equally successful. Osumi et al. (1991) identified a peroxisome proliferator responsive element (PPRE) in the 5'-upstream region of the rat peroxisomal AOX gene, between nucleotides -639 and -472. DNase I footprint analysis revealed two protein binding sites. In transient transcription assays using a rat hepatoma cell line, the isolated nucleotides from -578 to -553 conferred a liver-specific responsiveness to peroxisome proliferators. As will be presented in this thesis, our laboratory isolated a PPRE from the 5'-flanking region of the rat peroxisomal HD gene (Zhang et al., 1992; 1993). Comparison of its

sequence with the AOx PPRE revealed a common motif, a TGACCT consensus sequence, which appears as two direct repeats in the AOx PPRE and three direct repeats in the HD PPRE. This TGACCT code has been well characterized in many HREs as a half binding site for binding to transcription factors.

Dreyer et al. (1992) have shown that AOx PPRE specifically binds to three *Xenopus* nuclear receptors. These receptors are able to mediate the response of AOx PPRE to various peroxisome proliferators in the same fashion as the mouse PPAR. They are, therefore, named $xPPAR\alpha$, $xPPAR\beta$, and $xPPAR\gamma$. Like other members of the nuclear hormone receptor superfamily, 50% amino acid identity has been found among all these PPARs. A similar approach has also led to the cloning of PPARs from rat and human: rPPAR and hPPAR (Göttlicher et al., 1992; Schmidt et al., 1992). They are all closely related to mPPAR by amino acid sequence homology. Structurally, they all possess ligand and DNA binding domains. It has been reported that mRNAs of these xPPAR isoforms are expressed differentially: $xPPAR\alpha$ and $xPPAR\beta$, but not $xPPAR\gamma$, are expressed in oocytes and embryos. The $xPPAR\gamma$ is mainly in adipose tissue and kidneys, while $xPPAR\alpha$ and $xPPAR\beta$ appear ubiquitous (Dreyer, et al., 1993).

In our laboratory, all these receptors have been examined for their activity in activating the HD and AOx PPRE. MPPAR, rPPAR and $xPPAR\alpha$ were able to mediate a response of the PPREs to ciprofibrate

or Wy-14,643. Interestingly, $xPPAR\beta$ and $xPPAR\gamma$ were ineffective for the HD PPRE, but $xPPAR\gamma$ was effective for the AOx PPRE, indicating a differential specificity of these PPARs for a *cis* element (Marcus et al., 1993).

1.3.4 9-cis Retinoic Acid Receptor ($RXR\alpha$)

Kliwer et al. (1992a) have discovered that the 9-cis retinoic acid receptor, $RXR\alpha$, heterodimerizes with rPPAR. Both receptors are necessary for efficient factor-binding and transactivating AOx PPRE in response to a peroxisome proliferator or 9-cis retinoic acid. More importantly, neither receptor alone can function efficiently. Gearing et al. (1993) recently cloned a rat $RXR\alpha$ (r $RXR\alpha$) which showed a similar function as h $RXR\alpha$. It has been concluded that both PPAR and $RXR\alpha$ are required for a PPRE to respond to peroxisome proliferators.

This pathway was soon found to be shared by all the known peroxisome proliferators. For instance, a physiological concentration of fatty acids was able to regulate the peroxisomal β -oxidation via activation of PPARs in various systems (Göttlicher et al., 1992; Schmidt et al., 1992; Dreyer et al., 1993). The newly cloned mouse $RXR\beta$ and $xPPAR\alpha$ co-transfected into HeLa cells could be activated by 9-cis retinoic acid, long-chain unsaturated fatty acids, and clofibrate drugs (Keller et al., 1993). Thus, there clearly exists a convergence of the peroxisome proliferator- and retinoid-dependent signaling pathway on PPRE-like *cis* elements.

The convergence of the peroxisome proliferator signaling pathway with the 9-*cis* retinoic acid pathway but not the all-*trans* retinoic acid signaling pathway explains that retinoic acid as a peroxisome proliferator is weak, probably due to the necessity of its metabolic conversion into the RXR α ligand, 9-*cis* retinoic acid (Hertz et al., 1992; Farrants et al., 1993). The choice of 9-*cis* retinoic acid over all-*trans* retinoic acid may resemble the choice of one enantiomer of DEHP or clofibrate over the other. Recently, it was demonstrated that the interactions between mPPAR and the AOx-PPRE (-578 to -553) was stimulated mostly by the (S)-enantiomer of a peroxisome proliferator, leukotriene (Boie et al., 1993). It is, therefore, possible that peroxisome proliferators signal the pathway via PPAR and RXR α because of their structural resemblance to the endogenous amphipathic carboxylate RA or its metabolites, 9-*cis* retinoic acid, having a carboxyl function carried on a hydrophobic backbone (Hertz and Bar-Tana, 1992).

RXR α is a close relative of RAR by amino acid sequence homology (Mangelsdorf et al., 1990). Yet, RAR binds with high affinity to all-*trans* retinoic acid or all-*trans* didehydro-retinoic acid (*t*-ddRA) whereas RXR α prefers 9-*cis* retinoic acid or 9-*cis* didehydro-retinoic acid (9-*cis* ddRA), which are derived from retinoic acid in living cells (Heyman et al., 1992; Allenby et al., 1993). It has been known that RAR subtypes are expressed in distinct patterns of spatial and temporal distribution throughout development. RXR α is mostly expressed in the liver of a mature organism (Wolf and Phil,

1993).

Structural determination of the DNA-binding domain of hRXR α by nuclear magnetic spectroscopy revealed two zinc fingers and two α helices similar to the DNA binding domain of GR, ER, TR, RAR, and VD₃R (Katahira *et al.*, 1992; Lee *et al.*, 1993). However, a third helix was found in the DNA-binding domain of hRXR α . At least in solution, this additional helix mediates both protein-DNA and protein-protein interactions. RXR α has been shown to heterodimerize with RAR, TR, and VD₃R and thus to bind to diverse hormone-responsive elements (HREs) (Zhang *et al.*, 1992; Leid *et al.*, 1992; Kliewer *et al.*, 1992b). For *cis*-elements where homodimers bind poorly, heterodimers with RXR α show significantly higher binding efficiency and significantly higher transactivation activity (Zhang *et al.*, 1992; Kliewer *et al.*, 1992; Hallenbeck *et al.*, 1992).

Although the RXR α homodimer may itself be responsible for the regulation of certain genes (Mangelsdorf *et al.*, 1991), it is its role as a co-regulator that places it at the center of lipid metabolism (Umesono *et al.*, 1988; Zhang *et al.*, 1992; Hallenbeck *et al.*, 1992 and 1993; Allenby *et al.*, 1993; Wolf and Phil, 1993; Brockes, 1990). For mitochondria, the RXR α /RAR α dimer activates the gene encoding medium-chain acyl-CoA dehydrogenase, which catalyzes the initial rate-limiting step of fatty acid β -oxidation in response to retinoids (Raisher *et al.*, 1992). For the plasma lipid transport system, a retinoic acid responsive element (RARE) has

been isolated from the promoter of the gene encoding apolipoprotein AI, and it responds preferentially to RXR α (Rottman et al., 1991). A consensus PPRE sequence has also been reported to be present in 5'-upstream region of the gene encoding a cytosolic fatty acid binding protein (FABP) (Issemann et al., 1992). For peroxisomes, the RXR α /PPAR dimer mediates the response of PPREs in the peroxisomal AOX and HD genes to 9-*cis* retinoic acid and peroxisome proliferators (Kliwer et al., 1992a; Marcus et al., 1993). In a more recent study, transfection of mPPAR into several cell lines in the presence of clofibric acid or Wy-14,643 activated the luciferase reporter gene driven by a 5'-flanking sequence of the rabbit cytochrome P450IVA6 gene (a member of the P450IVA family), which led to the isolation of a new *cis*-element (Muerhoff et al., 1992). Through this P450IVA6 PPRE, PPAR and RXR α may play an essential role in the cooperation between the microsomal ω -hydroxylation and peroxisomal β -oxidation pathway.

1.4 This Project

This project has been designed towards the isolation of a PPRE located upstream of the gene encoding the rat peroxisomal HD and to investigate the specific interactions of this PPRE with nuclear proteins which trigger the response to peroxisome proliferators. The first goal was achieved by transient transfection studies. A core PPRE was identified as necessary and sufficient to confer peroxisome proliferator responsiveness. This PPRE contains three tandemly arranged consensus TGACCT motifs that are often present in many hormone-responsive elements. The sequence specificity of this HD PPRE was studied *in vitro* and *in vivo* by mutational analysis. A major protein-DNA complex was observed in mobility shift assays with a synthetic oligodeoxyribonucleotide containing the HD PPRE and nuclear extracts of rat hepatoma cells. This complex contained proteins that were able to react with antibodies against mPPAR, hRXR α , rHNF4, and hCOUP-TF1. A model of coordinated transcriptional regulation of the HD gene by multiple transcription factors will be discussed.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

The following is a list of critical reagents and the companies from which they were purchased. All chemicals and reagents were of the highest quality available.

ampicillin	Sigma Chemical Co.
ampicillin and streptomycin	Gibco Life Technologies, Inc.
bacto-agar	Difco Laboratories
Bio-Rad protein assay dye reagent	Bio-Rad Lab. (Canada) Ltd.
bovine serum albumin, DNase free	Pharmacia (Canada) Inc.
clofibrate	Sigma Chemical Co.
ciprofibrate	Sterling Drug
Co-enzyme A (yeast)	Sigma Chemical Co.
Dulbecco media	Gibco Life Technologies, Inc.
deoxyribonucleotides	Pharmacia (Canada) Inc.
diaminocyclohexane-N,N,N'- tetraacetic acid	Sigma Chemical Co.
dimethyl sulfate	Aldrich Chemical Co., Inc.
formic acid	BDH Inc.
glutamate	Gibco Life Technologies, Inc.
GTG agrose	FMC BioProducts
luciferin	Sigma Chemical Co.
molecular weight standards:	
(i) 1 kbp DNA ladder	Gibco/BRL Canada
(ii) Dalton Mark VII-L molecular weight markers for SDS-PAGE (14,000-66,000 Da)	Sigma Chemical Co.
(iii) phosphorylase b (rabbit muscle; 97,400 Da)	Sigma Chemical Co.
(iv) β -galactosidase (<i>E.coli</i> ; 116,000 Da)	Sigma Chemical Co.
nitrocellulose (pore size-0.45 μ m)	Schleicher & Schuell Inc.
nonidet P-40 (NP)	Sigma Chemical Co.
ovalbumin	Miles Laboratory (Pty) Ltd.
phenylmethylsufonyl fluoride	Boehringer Mannheim GmbH
piperidine	Aldrich Chemical Co., Inc.
poly (dI-dC) -poly(dI-dC)	Pharmacia (Canada) Inc.
protein A agrose	Bio-Rad Lab. (Canada) Ltd.
salmon sperm DNA (sonicated)	Promega Corp.

Sephadex G-50 (medium)	Pharmacia (Canada) Inc.
serum (calf, horse, or fetal calf)	Gibco Life Technologies, Inc.
sodium cacodylate	BDH Inc.
spermidine	Sigma Chemical Co.
spermine	Sigma Chemical Co.
trasyol (aprotinin)	Sigma Chemical Co.
tRNA (calf liver)	Boehringer Mannheim GmbH
Triton x-100	Sigma Chemical Co.
X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside)	Gibco/BRL Canada

2.1.2 Radioactive Chemicals

125 I-protein A (>30 mCi/mg total protein A, 0.1 μ Ci/ μ l)	Amersham Canada Ltd.
[γ -32P]ATP (4,500 Ci/mmol, 10 μ l)	Amersham Canada Ltd.
[α -32P]dATP (3,000 Ci/mmol, 10 μ l)	Amersham Canada Ltd.;
	ICN Biomedicals Inc.
L-[35S]-methionine (1151 Ci/mmol, 10 μ Ci/ μ l)	Dupont/NEN Canada Inc.

2.1.3 Enzymes

All enzymes were used according to the manufacturer's product data sheets unless otherwise specified in the method section.

DNA polymerase I (Klenow, <i>E. coli</i>)	New England Biolabs, Inc.
DNase I	Pharmacia (Canada) Inc.
proteinase K	Sigma Chemical Co.
restriction endonucleases	Gibco/BRL Canada;
	Pharmacia (Canada) Inc.;
	New England Biolabs, Inc.
T4 DNA ligase	Gibco/BRL Canada;
	Pharmacia (Canada) Inc.
T4 polynucleotide kinase	Pharmacia (Canada) Ltd.

2.1.4 Reagent Kits

<i>in vitro</i> transcription/translation	Promega Corp.
DNA sequenase, version 2.0	US Biochemical Corp.
ECL Western blotting system	Amersham International Inc.
Random primer DNA labeling kit	Gibco/BRL Canada

2.1.5 Antisera

Rabbit anti-hRXR α , anti-mPPAR, and anti-hCOUP-TF1 serum were raised in our laboratory by S. Marcus. From these sera, IgG was purified

by Affini-Gel Protein A Agarose following the manufacturer's procedure (Gibco/BRL Canada, Burlington, ON). The IgG fractions were pooled. They were neutralized with 6 volumes of 2 M Tris-HCl (pH 8.0), and dialyzed twice, each against 1,000 volumes of water, over a total period of 16 hr. This IgG preparation was lyophilized on a freeze dryer (Model FD-3-85A-MP, FTS Systems, Inc., Stone Ridge, NY). The pellet was dissolved in water to a protein concentration of 5 mg/ml. Rabbit anti-rHNF4 serum was a gift from Dr. F. M. Sladek, University California, Riverside, CA. It was directly used in mobility shift assays because of its limited quantity. The protein contents of the serum and IgG preparations were determined using the BioRad protein assay dye reagent following the product instructions (Bio-Rad Laboratories, Mississauga, ON).

2.2 Plasmids

2.2.1 *cdNA Plasmids Expressing Nuclear Transcription Factors*

The rPPAR α cDNA in pBluescriptIISK(+) was obtained from Dr. D. Noonan, Ligand Pharmaceuticals, San Diego, CA. S. Marcus excised the 2.6 kbp cDNA by *SpeI* and *EcoRV* digestion and inserted the fragment into the *XbaI* and *EcoRI* sites of a mammalian expression vector pRc/CMV (Invitrogen, San Diego, CA).

pSKXR3-1 containing the hRXR α cDNA was provided by Dr. R. M. Evans, Salk Institute, San Diego, CA. S. Marcus isolated the 1.8

kbp cDNA as an *EcoRI* fragment and ligated it into the *EcoRI* site of a mammalian expression vector, pSG5 (Green et al., 1988).

hCOUP-TF1 cDNA in pGEM7zf(+) was from Dr. M.J. Tsai, Baylor College of Medicine, Houston, TX. The 2.2 kbp cDNA was released as a *HindIII-XbaI* fragment and was inserted into the corresponding sites of pRc/CMV.

The 3.0 kbp cDNA of rHNF4 in the *BamHI* site of pSG5 was from Dr. F. M. Sladek, University of California, Riverside, CA. It was ready for mammalian cell transfections without further subcloning.

2.2.2 Reporter Plasmids Containing the Luciferase Gene

pCPSluc (Fig. 2.1) is a luciferase expression vector. It contains a 600 bp fragment of the proximal promoter of the gene encoding rat liver carbamoyl phosphate synthetase (CPS) (Howell et al., 1982). The *BamHI* site at the 5' end of this CPS promoter sequence was frequently used in this thesis for insertion of PPRE-containing fragments.

pHDluc5.8 was kindly provided by Dr. J.K. Reddy, Northwestern University Medical School, Chicago, IL. It contains 5.8 kbp of the upstream region of the rat peroxisomal HD gene and 22 bp downstream from the transcription start site. The HD promoter region was present as a *KpnI-ApaI* fragment in the luciferase expression vector pSVOALA5', linked immediately to the 5' end of the luciferase gene

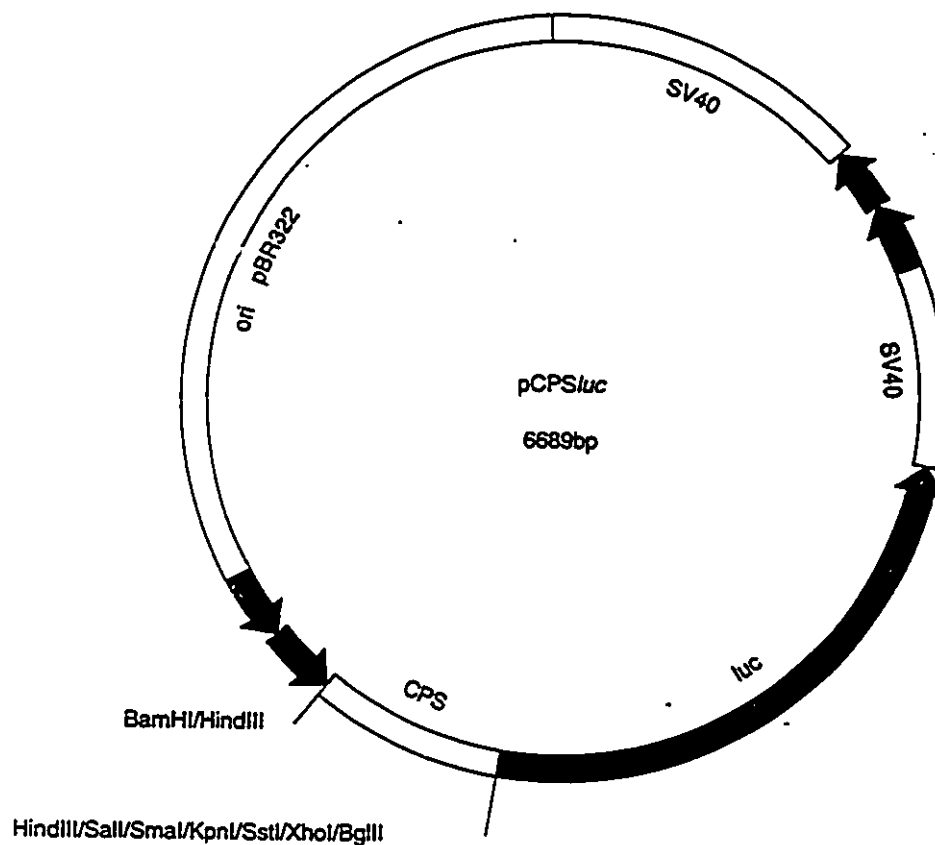


Fig. 2.1 pCPSluc luciferase expression vector. It is derived from pSVOALA5' (de Wet et al., 1987). The solid dark arrows represent short tandem repeats of SV40 polyadenylation signal sequence. The CPS proximal promoter (-600 to +1; Howell et al., 1989) was fused into the HindIII site by Dr. G. Shore's laboratory (McGill University, Montreal, QC).

(de Wet et al., 1987)

pSVOALA5', was isolated from pHDluc5.8 by KpnI and ApaI digestion. This linearized vector was used for construction of PPRE-driven reporter plasmids.

2.2.3 *pGEMHD5.8*

pGEMHD5.8 was constructed by S. Marcus in our laboratory. The 5.8 kbp of the HD promoter region was isolated from pHDluc5.8 as a KpnI-ApaI fragment and was inserted into the corresponding sites of a commercial vector, pGEM7zf(+) (Promega Corp., Madison, WI).

2.3 **Bacterial Amplification of Plasmid DNA**

2.3.1 *Introduction of Plasmids into Bacteria*

For a typical transformation, 3 μ l of a ligation mixture was added into 50 μ l of DH5 α competent cells (Gibco/BRL Canada, Burlington, ON). Transformation was performed according to the manufacturer's instructions. Five to ten bacterial colonies were picked and grown to late log phase in 3 ml LB with 100 μ g ampicillin/ml for isolation of plasmid DNA.

2.3.2 *Miniprep of Plasmid DNA*

Miniprep of plasmid DNA in small-scale bacterial culture followed the alkaline lysis protocol by Ausubel et al. (1992). The desired constructs were identified by restriction analysis, colony

hybridization, and DNA sequencing. An additional step of phenol extraction was necessary if the miniprep DNA was to be used in DNA sequencing.

2.3.3 *Large-Scale Isolation of Plasmid DNA*

To prepare plasmid DNA for mammalian cell transfections, 1.5 ml of a miniculture was used to inoculate 500 ml of LB containing 100 µg ampicillin/ml. After growth at 37°C with shaking for 16-20 hr, the plasmid DNA was isolated by alkaline lysis combined with either CsCl density gradient centrifugation (Ausubel et al., 1992), or chromatography on Qiagen columns (Qiagen, Inc., Chatsworth, CA). The plasmid DNA was dissolved in water and stored at -20°C. The DNA concentration was measured by absorbance at 260 nm using sonicated calf thymus DNA as a standard for which an absorbance of 1 at 260 nm indicated 50 µg/ml of double-stranded nucleic acid.

2.4 *Identification of Bacterial Colonies That Contain Recombinant Plasmids*

2.4.1 *Restriction Analysis*

Restriction analysis of small-scale preparations of plasmid DNA was performed to determine the presence of a restriction fragment and its orientation in a plasmid.

2.4.2 *Bacterial Colony Hybridization*

The *E. coli* colonies carrying the plasmid with a desired PCR-

synthesized fragment were identified by colony hybridization (Maniatis et al., 1982). The corresponding PCR product was purified and radiolabeled with [α - 32 P]dATP using a random primer DNA labeling kit and the manufacturer's procedure (Gibco/BRL Canada, Burlington, ON). Prehybridization was in a 65°C water bath for 3 hr and hybridization with the radiolabeled probe was at 42°C overnight. The nitrocellulose blots were washed in 1X SSC at 45-50°C three times for 15 min each. The shorter the probe, the lower was the washing temperature. Usually about 100 positive colonies were obtained after transformation of DH5 α cells. Eight of them would be picked and grown for plasmid DNA miniprep. Restriction digestions with appropriate enzymes were performed to determine the orientation of the insert. Plasmids with both orientations were used in mammalian cell transfections.

2.4.3 *Selection of Plasmid Recombinants by DNA Sequencing*

A primer, CPS UPS1, was synthesized and purified by B. Allore and D. Gooden, Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Its sequence, 5'- TTA TGT ACC TCT AAG TCT CTT -3', is complementary to the 5' end of the upper strand sequence of the CPS proximal promoter in pCPS $_{luc}$. It was used to sequence candidate plasmids in order to select the ones with the appropriate copy number and orientation of an oligodeoxyribonucleotide insert. The DNA sequencing was performed by using a sequenase version 2.0 kit and the protocol attached (US Biochemical Corp., Cleveland, OH).

2.5 Enzymatic Manipulation of DNA for Subcloning

2.5.1 Creation of Blunt-Ends

A total of 4.5 μ l of a blunt-end reaction mixture contained 0.5-1 μ g of DNA, 1 unit of T4 DNA polymerase, and 1.5 μ l of 5X KGB [5X KGB stock contains 100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.6), 10 mM magnesium acetate, 0.5 mM 2-mercaptoethanol, and 50 μ g BSA/ml (Ausubel et al., 1992)]. After incubation at 37°C for 2 min, 0.5 μ l of 0.5 mM dNTPs was added and the incubation was continued for another 10 min. DNA ligase could be directly added afterwards.

2.5.2 Phosphorylation

To phosphorylate the ends of an oligodeoxyribonucleotide, a 20 μ l of reaction mixture was made up of 1X linker-kinase buffer (Maniatis et al., 1982), 10 pmole of a double stranded oligodeoxyribonucleotide, and 2 units of T4 polynucleotide kinase (Pharmacia Canada Inc., Baie d'Urefé, QC). The mixture was incubated at 37°C for 30 min. The phosphorylated nucleotide was purified through a Sephadex G-50 spin column (Pharmacia Canada Inc., Baie d'Urefé, QC).

2.5.3 Dephosphorylation

The BamHI-linearized pCPSluc was treated with one unit of calf intestinal alkaline phosphatase per μ g of DNA in 1X NEB buffer 2 at 37°C for 1 hr (New England Biolabs, Inc., Mississauga, ON). The

product was extracted with phenol and desalted through a Sephadex G-50 spin column (Pharmacia Canada Inc., Baie d'Urefé, QC).

2.5.4 *Ligation of Blunt-Ended DNA*

For circularization of a plasmid with blunt ends, 1.5 μ l of 10 mM ATP and 1 μ l of 4.5 units/ μ l T4 DNA ligase (Pharmacia Canada Inc., Baie d'Urefé, QC) were directly added to the blunt-end reaction mixture. Water was added to bring the total volume to 15 μ l, so that the final reaction was in 1X KGB. The mixture was placed at 4°C for at least 16 hr before transformation.

2.5.5 *Ligation of Complementary Cohesive Termini*

For restricted DNA fragments, a typical ligation mixture contained 1 μ g of DNA in total, with a molar ratio of insert to vector approximately 5 to 1. The DNA was mixed with 1 unit of T4 DNA ligase (Gibco/BRL Canada, Mississauga, ON), 1 μ l of 10 mM ATP, and 1 μ l of 5X KGB in a total volume of 10 μ l. After incubation at room temperature for 16 hr, the plasmid was introduced into DH5 α cells.

PCR products were restricted to create cohesive ends. In a ligation mixture, a ratio of insert to vector was 1:1, w/w, with at least 200 ng of total DNA in 10 μ l of reaction volume.

To subclone a synthetic oligodeoxyribonucleotide into a reporter plasmid, 10 pmole of phosphorylated double-stranded oligonucleotide and 500 ng of dephosphorylated vector were ligated

in 0.5X KGB with 9 units of T4 DNA ligase in the presence of 1 μ g of calf thymus tRNA.

2.6 Construction of Plasmids for DNA Sequencing of the *NdeI*-*BglIII* Subregion of the HD Promoter

To sequence the *NdeI*-*BglIII* subfragment of the HD promoter, the plasmid with the Δ Xb/N fragment of the HD promoter (Fig. 3.1) in pGEM7zf(+) was used. Restriction analysis revealed the available sites from the 5' to 3' ends: *HindIII* in the vector, *KpnI* at the junction between vector and insert, a destroyed *NdeI*, *HindIII*, *PstI*, *BglIII*, and *ApaI*; simplified as H'-K-(N)-H-P-B-A. The N-H region was cloned into pGEM7zf(+) as an H'-H fragment making use of the *HindIII* site in the vector (H') which was immediate upstream of the insert. Both strands were sequenced using the universal or reverse M13 primer.

The H-P fragment was subcloned into the corresponding sites of pUC18 and pUC19. Both vectors have cloning sites *PstI* and *HindIII* but in opposite directions. With each vector, half the H-P fragment was sequenced from one end with the universal primer and the other half from the other end with the reverse primer. Overall, with the two vectors, both strands of the entire H-P fragment were sequenced.

The plasmid containing the P-B fragment was constructed by two-

steps: first, deletion of the B-A fragment, and then, deletion of the K-P fragment. Each step was followed by blunt-ending with T4 DNA polymerase and circularization with T4 DNA ligase. This plasmid allowed the sequencing of the entire P-B fragment from either orientation using the universal and reverse primers.

Approximately 100 bp downstream from the *Bgl*III site was also sequenced for the first time using a plasmid containing the B-A fragment. All the DNA sequencing reactions and the subsequent reading were conducted by Dr. R.A. Rachubinski. The sequenase version 2.0 kit was used and the manufacturer's standard procedure was followed. The entire sequence has been deposited in GenBank (accession no. M97197).

2.7 Fusion of Promoter Subfragments into Reporter Plasmids

2.7.1 Insertion of Deletion Mutants of the HD Promoter Region into *pSVOALA5'*

The 5.8 kbp of the HD promoter region was mapped mostly by S. Marcus using *pGEMED5.8*. Figure 3.1 illustrates the convenient sites for restriction deletion. *pGEMED5.8* was used to create deletions by double enzyme digestion in 1X KGB. *Sal*I was used in 1.5X KGB after digestion with a first enzyme. The plasmid DNA was then blunt-ended and recircularized. The Δ HD fragments were released by digestion with *Kpn*I and *Apa*I in 0.5X KGB and were isolated by GTG-agarose gel eletrophoresis and subsequent electroelution (Maniatis et al.,

1982). These fragments were finally inserted into the corresponding sites of pSVOALA5'.

2.7.2 *Fusion of Subfragments of the NdeI-BglII Region of the HD Promoter into pCPSluc*

For further fragmentation of the *NdeI-BglII* region, a plasmid with the fragment $\Delta Xb/N$ (Fig.3.1) in pGEM7zf(+), which was constructed while preparing deletion mutants of the HD promoter, was used. It contained the HD promoter fragment from its *NdeI* site to the transcription start region with a *BamHI* and a *BglII* site in between. An additional *BamHI* site in the vector was located a few base pairs upstream of the insert so that digestion of this plasmid with *BamHI* and *BglII* resulted in two fragments, 5'- *NdeI-BamHI* and 5'- *BamHI-BglII*. Both were directly subcloned into the *BamHI* site of the vector pCPSluc.

The plasmid with the *NdeI-BamHI* fragment contained a *HindIII* site in the middle. It was digested with *HindIII* with the CPS region discarded (Fig. 2.1) and then self-ligated. The remaining half of the HD subregion in the plasmid was released using the *BamHI* and *BglII* sites in the vector. This subfragment was then subcloned into the *BamHI* site of pCPSluc. The other half of the HD promoter subregion released by the *HindIII* digestion was first ligated to the large fragment of the *HindIII*-digested pCPSluc, isolated as a *BamHI-BglII* fragment using the sites in the vector, and then subcloned into the *BamHI* site of pCPSluc. Again, plasmids

with inserts in both orientations were used to transfect mammalian cells.

2.7.3 PCR Synthesis of HD Promoter Subfragments and Insertion of the PCR Products into pCPSluc

To further locate the HD PPRE within the *NdeI-HindIII* fragment (Fig. 3.2, e), PCR fragments were synthesized since there are no more convenient restriction sites available within this region. The following oligodeoxyribonucleotides were synthesized again by B. Allore and D. Gooden:

Forward primers:

(the underlined sequence is added to create a *BglII* site)

1. ATTAGATCTGTAATGGAATCTGATG (-3159)
2. ATTAGATCTGTGACCACTAG (-3040)
3. ATTAGATCTACATTTGAGTGCCCCG (-2928)

Reverse primers:

(the underlined sequence is added to create a *BamHI* site)

4. ATTGGATCCATTCTAAACTCACCCTGTA (-2751)
5. ATTGGATCCTGAAATTTATTTACTT (-2845)
6. ATTGGATCCCAAAGTCTCGGGGCA (-2900)

They were dissolved in water to appropriate concentrations

according to the equations:

$$1 \text{ OD}_{260} \text{ Unit} = 30 \text{ } \mu\text{g of Oligo}$$

$$\text{nmole of Oligo} = (\text{OD}_{260} \text{ Units} \times 90) / (\text{the length of the oligo})$$

Using combinations of these primers, subfragments of the HD promoter were amplified by PCR. The constructs g to l in Figure 3.2 were PCR products of primer pairs 1+4, 1+5, 1+6, 3+4, 2+4, and 2+5, respectively. In the same order were the annealing temperature of the PCR reactions: 48, 41, 49, 49, and 40°C, respectively. Routinely, 30 cycles were programmed each with melting at 92°C for 2 min, annealing at the appropriate temperature for 2 min, and polymerization at 72°C for 4 min. The template was a pGEM7zf(+) derivative containing the *NdeI-HindIII* fragment of the HD promoter. A total of 100 μl of PCR mixture contained 10 μl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl₂, 0.1% gelatin; Innis et al., 1990), 8 μl of 2.5 mM dNTP's, 20 pmole of each primer, 10 ng of the template plasmid, and 1 μl of Taq DNA polymerase (Boehringer-Mannheim GmbH, Montreal, QC). Since the primers have artificial restriction sites at their ends, the PCR products were digested with *BamHI* and *BglIII* and purified by GTG-agarose gel electrophoresis and subsequent electroelution. They were then inserted into the *BamHI* site of pCPS_{luc} under the conditions of cohesive-end ligation.

2.7.4. Insertion of Oligodeoxyribonucleotides into pCPSluc

The following oligodeoxyribonucleotides were also synthesized by B. Allore and D. Gooden:

Wild Type Oligodeoxyribonucleotides:

Upper and lower strands are synthesized separately; the lowercase letters represent the nucleotides added to create the 5' BamHI and 3' BglIII overhangs.

ACx (-583 to -544):

5'-gatCCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTA
GAAAGGGCTTGCACTCCAACAGGACCAGGGGAAAACGAtctag-5'

HD (-2956 to -2919):

5'-gatCCTCTCCTTTGACCTATTGAACTATTACCTACATTGGA
GAGAGGAACTGGATAACTTGATAATGGATGTAAACTctag-5'

Mutant HD Oligodeoxyribonucleotides:

The two strands were obtained separately and mixed if necessary. Only the upper strand sequences are listed. The 5'-ends of the lower strands are the same as those of the wild type HD oligonucleotide with a BglIII overhang added. The underlined nucleotides are mutated or deleted.

M1 5'-gatCCTCTCCTTTCACCTATTCAACTATTACCTACATTGGA

M2 5'-gatCCTCTCCTTTCAGGTATTCAAGTATTACCTACATTGGA

M3 5'-gatCCTCTCCTTTGACCTATTGAGTATTACCTACATTGGA

M4 5'-gatCCTCTCCTTTATTTTAAATTGAACTATTACCTACATTGA
M5 5'-gatCCTCTCCTTTGACCTATTGAACTACTATTCACATTGA
M6 5'-gatCCTCTCCTTTGACCT_ TTGAACTATTACCTACATTGA

Equimolar amounts of the complementary strands of an oligonucleotide were mixed in annealing buffer containing 0.05 M Tris-HCl (pH 8.0) and 0.01 M MgCl₂. Double stranded oligonucleotide was formed by heating the mixture at 90°C for 4 min and then cooling slowly to room temperature over a period of 1.5 hr. After treatment with T4 polynucleotide kinase, the oligonucleotide was ligated into the *Bam*HI site of pCPS*luc*.

To insert tandemly arranged multiple copies of an oligonucleotide into pCPS*luc*, 4.5 units of T4 DNA ligase (Pharmacia Canada Inc., Baie d'Urefé, QC) were directly added to the kinase reaction mixture and the incubation was continued at 37°C for at least 1 hr. After digestion with *Bam*HI and *Bgl*II, the ligation products were separated through a 12% polyacrylamide mini-gel in 1X TBE (Maniatis et al., 1982). A ladder of bands was visualized by ethidium bromide staining. The band with the size of three copies of the oligonucleotide was purified by electroelution and was ligated into the *Bam*HI site of pCPS*luc*.

2.8 Transient Mammalian Cell Transfection Assay

2.8.1 Cell Culture

All the cell lines were purchased from the American Type Culture Collection. Rat hepatoma cells, H4IIEC3, were cultured as monolayers in Dulbecco's modified minimal essential medium plus 10% horse serum and 5% fetal bovine serum. BSC40, Rat2, and HeLa cells were maintained in Dulbecco's modified minimal essential medium plus 10% calf serum before transfections, but charcoal-stripped serum was used in place of regular serum during transient transfections of these cells. This serum was prepared by a procedure from Dr. V. Giguere, the Hospital for Sick Children, Toronto, ON. First, dextran-coated charcoal (DCC) was made by stirring 25 g charcoal (Sigma Chemical Co., St. Louis, MO) and 2.5 g Dextran-70,000 in 1 l of 0.1 M Tris-HCl (pH 7.4) at 4°C overnight. Twenty ml of the mixture was centrifuged at 2,830 xg for 5-10 min. The DCC pellet was mixed with 100 ml of serum. The mixture was shaken at 56°C for 30 min. After stripping for a second round, the serum was filtered through 0.45 μ m and then 0.22 μ m sterile filterwares.

2.8.2 Transfections and Measurements of Luciferase Activity

Transient transfections were performed by a modification of the calcium phosphate precipitation method (Ausubel et al., 1989). H4IIEC3 cells were grown to 60-70% confluence in 10 cm dishes. A 2

ml solution was made up of 60 μg of a reporter plasmid DNA, 10 μg of sonicated salmon sperm DNA, and 250 μl of 2 M CaCl_2 . This solution was mixed dropwise with equal volume of a 2X HEPES-buffered saline [A litre of 2X HBS contains 16.4 g NaCl, 11.9 g HEPES-acid, and 0.21 g Na_2HPO_4 with an overall pH 7.12]. A 20 min incubation at room temperature was followed to allow precipitation to occur. The suspension was divided into 1 ml aliquots. Each aliquot was added dropwise to cells incubated in the presence of 10 ml of freshly added medium containing either 0.5 mM ciprofibrate (100 mM stock solution in dimethyl sulfoxide) or 0.5% dimethyl sulfoxide alone. After incubation at 37°C for 16 hr, the medium was replaced with fresh medium supplemented with ciprofibrate or the solvent. The incubation was continued for another 24 hr. To harvest, cells were washed with phosphate-buffered saline (PBS), scraped into microcentrifuge tubes, and lysed in 100 μl of lysis buffer [25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% (w/v) glycerol, 1% (w/v) Triton X-100]. Cell debris was removed by centrifugation at 4°C for 15 min. The supernatant was assayed for luciferase activity with a luciferase assay kit (Promega Corp., Madison, WI) and a luminometer (model 1253, Bio-Orbit Oy, Turku, Finland) following the manufacturer's protocol. When necessary, protein content of the cell lysates was determined by the method of Bradford (Bio-Rad Laboratories, Mississauga, ON).

Transfections of BSC40, HeLa, and Rat2 cells followed

essentially the same procedure except 10 μ g of the reporter plasmid containing a PPRE and 4 μ g of the plasmid containing a nuclear receptor cDNA were added to each plate. The corresponding empty expression vector(s) were added to keep the dosage of the effector plasmids constant. Five hr before the plasmid DNA was added, the cells were subjected to a medium made up of phenol red-free Dulbecco medium and 10% charcoal-stripped fetal calf serum. This medium was used throughout the rest of the experiment.

2.9 Preparation of Protein Extracts for DNA-Binding Assays

2.9.1 Preparation of Nuclear Extracts from Cultured Cells

For DNase I footprint analysis, methylation interference analysis, and mobility shift analysis, the nuclear extracts of H4IIEC3, HeLa, BSC40, and Rat2 cells were prepared by Dignam's procedure (Dignam et al., 1983) with slight modification by Dr. J. Capone's laboratory. Ten confluent plates of cells were collected, washed twice with PBS, and incubated in buffer A on ice for 10 min (Dignam et al., 1983). The cells were lysed by incubation on ice for 30 min with 2 ml of buffer A containing 0.5% NP40. The nuclei were pelleted by centrifugation at 2,200 xg. The rest of the procedure followed exactly that of Dignam. The nuclear proteins obtained were dialysed twice against 1 liter of buffer D at 4 °C overnight (Dignam et al., 1983). Nuclear extracts were also prepared from H4IIEC3 cells cultured for 48 hr in the presence of either 0.5 mM ciprofibrate or 0.5% dimethyl sulfoxide. All extracts were stored

at -70°C.

2.9.2 Detergent-Free Preparation of Cytosolic and Nuclear Extracts

For the last part of this thesis, H4IIEC3 cells were also fractionated into cytosol and nuclear fractions. The preparation followed exactly the method of Dignam (Dignam et al., 1983) without detergent or any other changes.

2.9.3 In Vitro Transcription/Translation

In vitro transcription of cDNAs encoding rPPAR, hRXR α , rHNF4, or hCOUP-TF1 and subsequent translation in rabbit reticulocyte lysate were carried out using a commercial kit (Promega Corp., Madison, WI). The plasmids with cDNAs of rPPAR, hRXR α , hCOUP-TF1, and rHNF4 were linearized with BamHI, HindIII, EcoRI, and XhoI, respectively, and the cDNAs were transcribed by T7, T3, Sp6, and T3 RNA polymerase, respectively. The proteins to be used in electrophoretic mobility shift assays were synthesized with unlabeled methionine. The proteins were also produced separately in the presence of ³⁵S-methionine in order to check the quality of the products by SDS-PAGE.

2.10 Analysis of DNA-Protein Interactions

2.10.1 Mobility Shift DNA-Binding Analysis

Two probes of different size were used. A 196-bp subfragment of the

minimal HD PPRE fragment (-3040 to -2845) was synthesized by PCR. After restriction digestion with *Bam*HI and *Bgl*III to create overhangs, 0.5 μ g of the PCR product was mixed with 50 μ Ci [α -³²P]dATP, 1 unit of Klenow fragment, and 1 μ l of 0.5 mM dNTPs (0.5 mM each of dGTP, dTTP, and dCTP) in 1X KGB. The mixture was incubated at 37°C for at least 2 hr. The probe DNA was purified by 2% GTG-agarose gel electrophoresis and electroelution.

To radiolabel the oligodeoxyribonucleotides, the complementary strands were annealed to a final concentration of 10 pmoles/ μ l. Fifteen pmoles of a double-stranded oligonucleotide was mixed with 100 μ Ci of [α -³²P]dATP, 1 unit of Klenow fragment, and 1 μ l of dNTP mixture containing 0.5 mM each of dGTP, dCTP, and dTTP. The mixture was incubated at 37°C for 2 hr. The probe was purified by 4% GTG-agarose gel electrophoresis onto a piece of DEAE paper and was eluted at 65°C for over 1 hr into a DEAE elution buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1M NaCl] (Maniatis et al., 1982). The eluate was transferred to a new Eppendorf tube and centrifuged at 4°C for 15 min to remove the residual DEAE paper and agarose gel. The probe DNA was precipitated by ethanol with the addition of 4 μ l of 1 M MgCl₂. The pellet was washed with 70% ethanol, dissolved in 100 μ l of water, and stored at -20°C. As a control probe, a synthetic oligodeoxyribonucleotide, 5'-GATCCCGTGCATGCTAATGATATTCT, was also radiolabeled and used in mobility shift assays. The underlined nucleotides represent the binding site for the ubiquitous nuclear transcription factor, Oct-1 (Werstuck and

Capone, 1993).

A total of 20 μ l of a protein-binding mixture contained 30,000 cpm of a radiolabeled probe, 10 mM HEPES (pH 7.9), 120 mM NaCl, 1 mM EDTA, 7% (v/v) glycerol, 5 μ g of bovine serum albumin, 4 μ g of nonspecific competitor DNA [a 1:1 mixture of poly(dI-dC)·poly(dI-dC) and sonicated salmon sperm DNA], 150 μ M phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μ g of nuclear protein prepared from a monolayer cell culture. After incubation at 30°C for 20 min, electrophoresis was performed at 4°C on a pre-equilibrated 3.5% polyacrylamide gel (30:1, w/w, acrylamide/N,N'-methylenebisacrylamide). The running buffer contained 22 mM Tris-base, 22 mM boric acid, 1 mM EDTA. After approximately 3 hr, the gel was dried and exposed to XAR-5 Kodak film at -70°C with an intensifying screen.

In competition mobility shift analysis, unlabeled competitor DNA was added, where indicated, in a 50- and 100- molar excess relative to the radiolabeled probe. A negative control competitor was provided by G. Eitzen of our laboratory. It contains the upstream region (-775 to -613) of the yeast gene encoding a version of peroxisomal HD and has little homology with the rat HD promoter sequence.

Mobility shift analysis was also done in the presence of antibodies against nuclear transcription factors. In these

experiments, 5 μg of IgG purified from an antiserum was preincubated at 30°C for 5 min with cellular protein extracts in the mobility shift binding buffer before the probe was added. Since there was only limited amount of anti-rHNF serum available, 1 μl serum (approximately 30 μg of protein) was added in a reaction without purification.

2.10.2 DNase I Footprint Analysis

The probe DNA was a 196-bp fragment that contained the minimal HD PPRE. It was synthesized by PCR and inserted into the *Bam*HI site of pSP73 (Promega Corp., Madison, WI). For upper strand labeling, the recombinant plasmid was linearized with *Bam*HI and end-labeled with [α -³²P]dATP and the Klenow fragment. The reaction was terminated by extraction with buffer-saturated phenol. The radiolabeled probe was separated from unincorporated nucleotides on a Sephadex G-50 spin column (Pharmacia Canada, Inc., Baie d'Urefé, QC). The PPRE-containing fragment was excised with *Hind*III and recovered by GTG-agarose gel electrophoresis and electroelution. For lower strand labeling, the same procedure was followed except that the recombinant plasmid was first linearized with *Hind*III and, once labeled, it was digested with *Bam*HI.

The protein-binding reaction was carried out in a total volume of 100 μl by mixing 20,000 cpm of a double-stranded DNA probe with 100 μg of nuclear extract (or 100 μg of bovine serum albumin as a control), and 1 μg of nonspecific competitor poly(dI-dC)·poly(dI-

dC) in a buffer containing 10 mM HEPES (pH 7.9), 120 mM NaCl, 1 mM EDTA, 7% (v/v) glycerol, 0.15 mM phenylmethylsulfonyl fluoride, and 1 mM DTT. After incubation on ice for 15 min, DNase I was added, 1.76 μg for samples containing nuclear extract and 0.44 μg for control samples containing bovine serum albumin. The incubation was continued on ice for exactly 3 min. The reaction was terminated by addition of 200 μl of a stop buffer [100 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 mM NaCl, 0.1% sodium dodecyl sulfate] plus 10 μg of tRNA and 100 μg of proteinase K. After incubation at 37°C for another 30 min. The nucleic acid product was extracted with buffer-saturated phenol and precipitated with ethanol. The pellet was dissolved in a formamide-containing buffer. Approximately 10,000 Cerenkov counts of each sample were subjected to electrophoresis on a 4% polyacrylamide standard DNA-sequencing gel. The dried gel was exposed to XAR-5 Kodak film at -70°C with an intensifying screen.

G reactions of chemical sequencing of the same probes were performed to provide a ladder of nucleotide positions (Ausubel et al., 1989). Briefly, the probe end-labeled at one strand was partially methylated with 1 μl of dimethyl sulfate (DMS) at 20°C for 1 min in 200 μl of DMS buffer [50 mM sodium cacodylate (pH 8.0), 1 mM EDTA, and 1 μg tRNA]. The reaction was stopped with 50 μl of a solution containing 1 M 2-mercaptoethanol and 1.5 M sodium acetate (pH 7.0). The nucleic acid was precipitated with 750 μl of ethanol precooled to -70°C. The pellet was dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. This methylated probe was cleaved with

10% (v/v) piperidine at 90°C for 30 min. The cleavage was stopped by quick freezing and lyophilizing. To remove the residual piperidine, the cleaved probe was resuspended in 50 μ l of water and dried by vacuum. This last step was repeated at least three times. The Cerenkov counts of the final dried product was measured. Approximately 10,000 cpm was loaded into each lane.

2.10.3 *Methylation Interference Analysis*

The synthetic oligodeoxyribonucleotides containing the wild type HD PPRE or the AOx PPRE were used as probes. Each single-stranded oligonucleotide of 10 pmole was radiolabeled at its 5' end with 100 μ Ci of [γ -³²P]ATP by 2 units of T4 polynucleotide kinase in 1X linker ligation buffer (Maniatis et al., 1982). The labeled strand was annealed with an equimolar amount of the unlabeled complementary strand at 90°C for 4 min in 5 mM Tris-HCl (pH 8.0) and 1 mM MgCl₂, followed by cooling slowly to room temperature over a period of 1.5 hr.

The double stranded probe was partially methylated by the G reaction of chemical sequencing. The methylated probe was used in a standard mobility shift assay with H4IEC3 nuclear extract. Visualized by autoradiography, the bound and the free probes were separately excised from the gel, embedded in a 1% agarose gel, electrophoresed in 1X TBE onto a piece of DEAE paper, and subsequently eluted at 65°C for at least 2 hr in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 M NaCl. The recovered DNA was cleaved with

10% (v/v) piperidine at 90°C for 30 min. The piperidine was evaporated in a vacuum chamber. The dried sample was dissolved in 50 μ l of water and dried again under vacuum. This last step was repeated twice more. After the Cerenkov counts were measured, the pellet was dissolved in formamide-containing buffer and subjected to electrophoresis on a 20% polyacrylamide DNA-sequencing gel. Approximately 10,000 Cerenkov counts of each sample were loaded in each lane. The dried gel was exposed to Kodak XAR-5 film at -70°C with an intensifying screen.

The G reaction of chemical sequencing alone was also performed as in the DNase I footprint analysis. For the G+A reaction, the annealed probe of 10,000 counts was incubated with 25 μ l of formic acid at 30°C for 4 min. The reaction was stopped by adding 200 μ l of the DMS stop buffer. The rest of the protocol followed the G reaction procedure.

2.11 Analysis of Proteins

2.11.1 Preparation of Nuclear Extracts from Rat Liver

Adult Sprague Dawley rats of 120 g body weight were fed a diet of Purina rat chow containing 0.5% w/w clofibrate for 15 days, while untreated rats were fed a diet of Purina rat chow treated with ether, the solvent of clofibrate. The chow was rolled into balls and was baked at 60°C until no residual smell of ether was left. Rat liver of approximately 12 g was homogenized with a B pestle

homogenizer in 90 ml of homogenization buffer [10 mM HEPES (pH 7.5), 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2.2 M sucrose, and 5% glycerol]. The homogenate was aliquoted into three SW28 tubes (Beckman Instruments, Inc., Palo Alto, CA) on top of 10 ml of the same buffer and centrifuged at 4°C for 60 min at 110,000 xg in a L8-M ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The nuclear pellet was resuspended in a buffer made up of glycerol and cushion buffer at a ratio of 1 to 9. The cushion buffer was made up of homogenization buffer containing 2 M sucrose and 10% glycerol. The nuclei were pelleted again as before and were resuspended in 10 ml of lysis buffer [10 mM HEPES (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 3 mM MgCl₂, 1mM DTT, 0.1 mM PMSF, 1% Trasylol]. After it was homogenized with an A pestle homogenizer, 0.1 volume of 4M (NH₄)₂SO₄ was added. The mixture was centrifuged in a Ti50.2 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 110,000 xg for 30 min. The supernatant was immediately transferred to a new Ti50.2 tube. For every ml of supernatant, 0.3 g of (NH₄)₂SO₄ was added. The mixture was incubated on ice for 1 hr and then centrifuged at 150,000 xg for 20 min. The nuclear protein was resuspended in 1.0 ml of dialysis buffer [25 mM HEPES (pH 7.6), 0.1 mM EDTA, 100 mM KCl, 15% glycerol, and 1 mM DTT] and dialysed twice at 4°C, each time for 4 hr against 100 volumes of dialysis buffer. The recovered dialysate was centrifuged in a microcentrifuge at 4°C for 5 min. The supernatant was frozen in liquid nitrogen and stored at -80°C.

2.11.2 *Western Blotting Analysis*

To detect the presence of RXR α in nuclear lysates, Western blotting analysis was performed using the ECL Western Blotting Detection System (Amersham Canada Ltd., Oakville, ON). Approximately 250 μ g of a liver nuclear lysate from clofibrate-treated or non-treated rats was electrophoresed on a 10% SDS-polyacrylamide gel. The protein was transferred onto nitrocellulose in electrophoretic transfer solution (20 mM Tris-Base, 150 mM glycine, and 20% methanol) at 100 mA in a transfer apparatus (Bio-Rad Transblot). Electrophoresis and the subsequent blotting followed the protocol of Maniatis (Maniatis et al. 1982). After 13 hr, a Western blotting kit (Biorad Lab, Ltd. Canada, Mississauga, ON) was used to treat the blot with milk, then the anti-RXR α (20 μ g of purified IgG in 10 ml of milk), and finally the 125 I-protein A, each step following the manufacturer's instructions. The blot was air-dried on a filter paper and exposed to a XAR-5 Kodak film for 13 hr at room temperature.

RESULTS AND DISCUSSION

Alteration of gene expression by peroxisome proliferators is the major event that associates with an increase in the peroxisome population in hepatocytes and possibly to the ultimate hepatic tumorigenesis in rodents (Reddy and Lalwani, 1988). To understand the mechanisms of this altered gene expression, this project was designed to identify and characterize the promoter elements and the transcription factors responsible for the peroxisome proliferator-induced overexpression of the gene encoding the rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD).

3.1 Identification of a Peroxisome Proliferator Responsive Element (PPRE) in the Upstream Region of the HD Gene

3.1.1 A *Cis* Element Responsive to Ciprofibrate is Present in the Upstream Region of the Rat HD Gene

Treatment of H4IIEC3 cells with ciprofibrate resulted in a marked peroxisome proliferation and a concomitant transcriptional induction of the genes encoding peroxisomal β -oxidation enzymes (Osumi et al., 1991). To launch this project, Dr. J. Capone and Dr. R.A. Rachubinski had confirmed these results by Western blotting analysis. I joined the initial work by subcloning the 5.8 kbp of the HD promoter region into a mammalian expression vector, pRSVCat, which has a chloramphenicol acetyl transferase (CAT) gene as the

reporter gene. Deletion of the upper 3-kbp portion of this HD promoter was achieved by *KpnI* and *BglII* digestion followed by an blunt-end reaction and religation. The remaining sequence from *BglII* downstream to the transcription start site was also inserted into pCAT. Dr. J. Capone assayed the promoter efficiency of these constructs by introducing plasmids containing the full length HD promoter or the *KpnI/BglII* deletion mutant into H4IIEC3 cells and measuring the CAT activity of the cell lysate. The full length HD promoter conferred a responsiveness to ciprofibrate whereas the *KpnI/BglII* deletion resulted in a loss of the responsiveness, clearly indicating the presence of a peroxisome proliferator responsive element (PPRE) within the *KpnI-BglII* region (data not shown).

The HD promoter and its subfragments were also subcloned into pSVOALA5' which carried a luciferase gene as the reporter gene. Transfections of H4IIEC3 cells with pHDluc5.8, which contained the entire 5.8 kbp upstream sequence of the HD gene in pSVOALA5', resulted in a low level of luciferase activity (~0.5-1 light units). This activity was induced 5-10 fold by ciprofibrate, showing again that sequences mediating the response to ciprofibrate were present in this 5.8 kbp HD promoter region (Fig. 3.1, WT). Ciprofibrate treatment had no effect on the expression of pHDluc5.8 in BSC40 cells, a monkey kidney cell line, indicating that the ciprofibrate responsiveness of the HD gene was specific to liver cells (data not shown). This was consistent with the liver

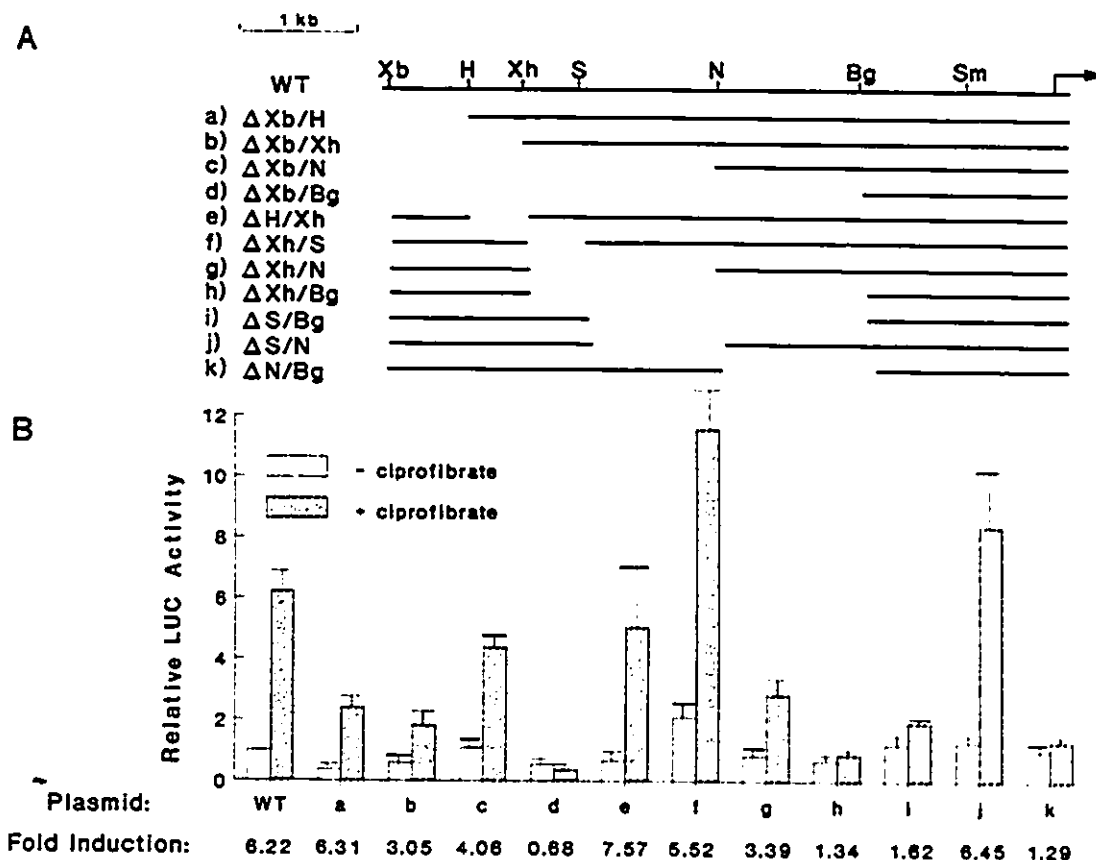


Fig. 3.1 A *cis*-acting element in the HD promoter region is necessary for ciprofibrate-responsiveness. (A) The restriction map of the 5.8 kbp HD promoter region (WT) and the diagram of the deletion mutants (Δ) from a to k. The letters designate the restriction sites between which the region is deleted. From the 5'-end: Xb-XbI, H-HpaI, Xh-XhoI, S-SalI, N-NdeI, Bg-BglII, Sm-SmaI. (B) The relative luciferase (LUC) activity of the cell lysate from H4IIEC3 cells transfected with plasmids containing the wild type HD promoter or deletion mutants, plasmids a - k, in the absence (empty bar) or presence (shaded bar) of ciprofibrate. Results of three or more repeated assays are averaged and normalized (\pm SEM) on the basis of the activity of the WT HD promoter without ciprofibrate which is usually 0.5-1.5 integrated light units. Fold induction is the ratio of the relative luciferase activity of the ciprofibrate-treated samples (+ciprofibrate) over that of the non-treated ones (-ciprofibrate). Background luciferase activity obtained from the empty vector, pSVOALA5' is close to zero.

specificity of the induction of endogenous HD by peroxisome proliferators (Osumi et al., 1991). In addition, luciferase expression from empty vectors, pRSVcat or pSVOALΔ5', as the negative controls for the experiment, was not increased by ciprofibrate in either H4IIEC3 or BSC40 cells (data not shown).

The restriction sites in the 5.8 kbp upstream region of the HD gene (Fig. 3.1.A) was used to create deletion mutants for a preliminary localization of PPREs. Due to the relatively low transfection efficiency of H4IIEC3 cells, all transfections were carried out in duplicate and repeated at least four times. The wild-type plasmid, pHDluc5.8, was used as a positive control each time and the data were normalized by protein concentrations of the cell lysates. Each construct was repeatedly assayed by both S. Marcus and myself. The same strategy was used to assay all the constructs in Figures 3.1 and 3.2. The absolute level of ciprofibrate induction varied as a function of the background luciferase activity of each construct without the influence of ciprofibrate, which varied up to 2-fold. However, the induction ratios remained fairly constant. The means of the induction ratios from my experiments and those of S. Marcus were consistent.

As is shown in Figure 3.1.B, plasmids a, b, and c, sequential end-deletion of the HD promoter XbaI site at the 5'-end down to the HpaI, XhoI, or NdeI site at position -3178 (ΔXb/H, ΔXb/Xh, and ΔXb/N, respectively), had little effect on ciprofibrate

inducibility, which ranged from 3- to 6-fold for the deletion constructs versus 6-fold for the wild-type HD promoter (WT). So the region from the 5'-end to -3178 does not seem to contain any major PPRE. It was a deletion from the 5'-end of the promoter region further down to the *Bgl*III site at -1623 that completely abolished inducibility (plasmid d with deletion Δ Xb/Bg). Together, data from these constructs indicate that a *cis* element was present between the *Nde*I and *Bgl*III sites. Its presence is necessary for ciprofibrate responsiveness.

The positive role of this *Nde*I-*Bgl*III region in mediating ciprofibrate induction was also demonstrated by a series of internal deletions. Every deletion that deleted this region resulted in a loss of responsiveness to ciprofibrate (Fig. 3.1, plasmids h, i, and k with deletions Δ Xh/Bg, Δ S/Bg, and Δ N/Bg, respectively). In contrast, all the fragments that retained this region were inducible (plasmids e, f, g, and j with deletions Δ H/Xh, Δ Xh/S, Δ Xh/N, and Δ S/N, respectively). The smallest deletion that leads to a complete loss of inducibility is the deletion between the *Nde*I and *Bgl*III sites (Δ N/Bg as in plasmid k). It is, therefore, concluded that a major ciprofibrate-responsive element lies between *Nde*I and *Bgl*III. If any additional PPREs exist upstream of the *Nde*I site, they are unable to function in the absence of the important sequences between the *Nde*I and *Bgl*III sites.

3.1.2 *The HD PPRE Fragment Confers Ciprofibrate-Responsiveness onto a Heterologous Promoter*

In order to investigate whether the region between the *NdeI* and *BglIII* sites (-3178 to -1623) was sufficient for ciprofibrate-mediated induction by itself, and to further locate the PPRE within this region, the *NdeI*-*BglIII* region was restricted into three smaller fragments using the internal *HindIII* and *BamHI* sites at nucleotides -2676 and -2281, respectively. They were inserted into the *BamHI* site of pCPS_{luc}, linked to the 5' end of the minimal promoter of the gene encoding carbamoyl phosphate synthetase (CPS). The CPS gene is also specifically expressed in hepatocytes but it is unresponsive to peroxisome proliferators. In this vector, the CPS promoter is linked directly to the luciferase gene.

The region between the *NdeI* and *BamHI* sites conferred inducibility when it was linked to the CPS promoter (Fig. 3.2, plasmid a). The background expression of the empty pCPS_{luc} in the absence of ciprofibrate was not significantly affected by any subregion of the HD promoter (data not shown). This *NdeI*-*BamHI* fragment, therefore, contains a positive PPRE that functions independently of other subregions of the 5.8 kbp HD promoter. A similar approach led to the further localization of the putative PPRE between *NdeI* and *HindIII* sites (-3178 to -2676, compare plasmids e and f). This PPRE displayed enhancer-like properties. It functioned in a position- and orientation-independent manner (Fig. 3.2, plasmids a and b). All these HD subfragments were subcloned

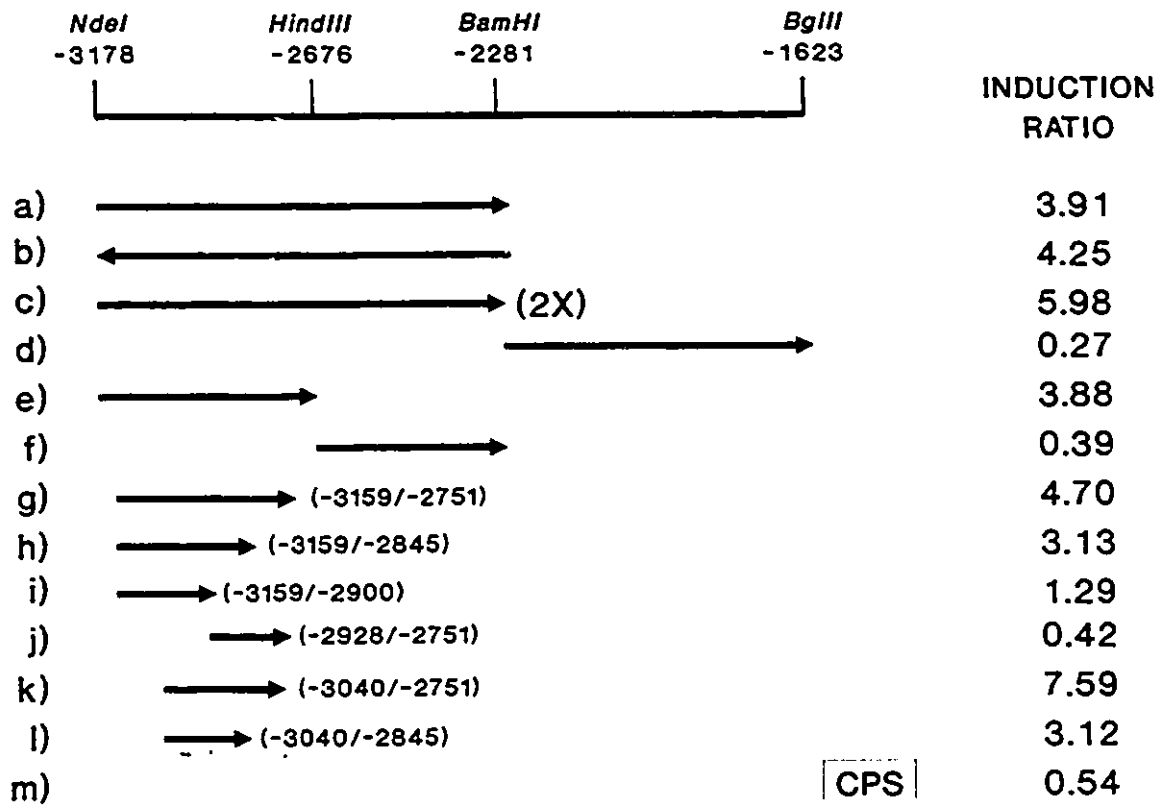


Fig. 3.2 A minimal PPRE is located between -3040 and -2845 in the upstream region of the HD gene. The *NdeI*-*BglIII* region of the HD promoter as illustrated on the top and its subfragments, a - m, were inserted into the *BamHI* site of pCPSluc linked to the 5'-end of the CPS minimal promoter (CPS). The arrows point to the orientation of the inserts. The construct c contains two copies of the insert, head to tail ligated. The numbers in parentheses correspond to the positions of the first/last nucleotides. Fragments a - f were obtained by restriction digestion; g - m, by PCR. Results of repeated transfections of a construct are averaged and normalized against those from the empty pCPSluc without the addition of ciprofibrate. The induction ratio is the luciferase activity of ciprofibrate-treated cells over that of the untreated cells. pHDluc5.8 (Fig. 3.1., WT) was used as a positive control (data not shown). pCPSluc is a negative control which, in the absence of the drug, gives integrated light units ranging from 0.2 to 1.

into pCPS_{luc} in both orientations and the constructs with both orientations were assayed. The results were consistent (data not shown). Two copies of this region led to a higher level of induction relative to a single copy (compare plasmids c and a), which is also a common feature of enhancers in inducible genes (Strähle et al., 1988).

The nucleotide sequence of the entire *Nde*I-*Bam*HI fragment had been obtained at this point (Fig.3.3). This enabled us to design PCR primers to amplify and subclone smaller fragments into pCPS_{luc} to continue the search for the PPRE (also in Fig. 3.2, plasmids h - m). A 196-bp fragment between -3040 and -2845 rendered a significant induction by ciprofibrate (plasmid l). This fragment is the smallest that retained full ciprofibrate inducibility. Plasmid j shares 84 bp with the 3'-end of this 196-bp fragment in plasmid l. It was, however, unresponsive to ciprofibrate and, like other unresponsive plasmids (plasmids d, f, and m), it showed reduced activity in the presence of ciprofibrate. Plasmid i shared 141 bp with the 5'-end of the 196-bp fragment in plasmid l. It was induced only to 1.3-fold. The region overlapped by plasmids i and j may, therefore, contain sequences important for a maximal ciprofibrate induction. These results have led to the conclusion that the 196-bp fragment in plasmid l contains a minimal PPRE which renders a maximal response upon ciprofibrate treatment. The elements contained in this 196-bp fragment are, therefore, called the minimal HD PPRE, hereafter.

*Nde*I
 -3178
 CATATGTTGG CTCACAACCA TCTGTAATGG AATCTGATGC CCTCTTCTGG TGTGCTGAA
 GTATACCACC GAGTGTGGT AGACATTACC TTAGACTAAG GGAGAAGACC ACACGACTT

-3118
 GACAGCTACA GTGTACTCAT ATACATAAAT AAATCTTAAA AAAGAAAAAA GGAGGAAGAA
 CTCTGCATGT CACATGAGTA TATGTAITTA TTTAGAATTT TTTCTTTTTT CCTCCTTCTT

-3058
 AATCACTAAA ATTTAGCACT GTGACCACTA GATGTCAGGA GGTAAAGTGT GAGTCTGCGT
 TTACTCATT TAAATGGTCA CACTGGTAT CTACAGTCTT CCATTTTACA CTCAGACCGA
 ←————— ←—————

-2998
 CTGTTTATCT CTGCTCCGCA CCGCACCGCA CCGCTACCGG CAGCCTCTGT TTTGCTATT
 GACAAAAGA CAGCAGGGCT CCGCTCCGCT CCGCATCGGG CTGGAGAGCA AACTGGATAA
 ●—————●

-2938
 GATCTATTAC CTGACTTGA GTGCCCCGAG ACTTTGGGAG CTGATAGCTC TTAACAATT
 CTGATAATC GATTAAGT CACGGGGCTC TAAACCTC CACTATCCAG AATTGTAAA
 ●—————●

-2878
CTGAGGAG TAAAAAGTA AATAAATTC AGGAATAATT CTAGGCTTTT CTTTCCACAT
 CGACTGCTC ATTTTTCAT TTATTTAAG TCCTTATTA GATCCGAAA GAAAGGTCTA

-2818
 CTAGTCCCT GCCCTTTTAC CTATTAAG AACTTCTTTT TAACTACAG TGGTGAGTTT
 GATCAGGGA CCGGAAAATC GATAATTTT TGAAGAAA ATTCATGTC ACCACTCAA

-2758
 AGAATCGAAA GATGAAACCA TAGAGAGGAA TTTACAGAAA ACGGAGAAA AGTCATTACA
 TCTTACCCTT CTACTTTGCT ATCTCTCCTT AAATGCTTTT TGCCCTTTT TCAGTAATGT

-2698
 TCCCTCTTC TTGCATGTTT *Hind*III TGAGCTTCA TCTGTATAGC CCAGGGTGGT CTCAAACTCA
 AGGGAACG AAGTACAAA ACTTCAAGT AGACATATCG GGTCCACCA GAGTTTGA

-2638
 GAGTCATCT CCTGCCTCTG CCTCCTGAGT ATTGAATTC CAGCCTTGA CCACCACAGT
 GTCAGTAGGA GGACGGAGAC GGAGGACTCA TAACTTTAA CTGGAACTT GGTGGTCTA

-2578
 ACCATAACAC CATGTTGAGC AGAGCCAATC CCAAAACACC GTGGTTTGA GACTGGACTT
 TGGTATTGTG GTACAAGTGG TCTGGTTAC GCTTTTGTG CACCAAACT CTCACCTGAA

-2518
 ACTGTAAAAC ACAAGATGA AACAGTGATC AAAACGGTCA CATATTAATA TTGTTTTAGT
 TGACATTTT TCTTACTT TTGTCACTAG TTTTGCCACT GTATAATTAT AACAAAATCA

-2458
 ATATTCTTAC TTTTCTGCA TGCTTGA AAA CTTACATAAT GCAAAGTTAA AATGACAAGC
 TATAAGATC AAAACCACCT ACCAACTTTT GAATGTATTA CGTTTCAATT TTACTCTTGC

-2398
 GTCATTAGTG ATCCATGAAT ATTTTCTTTC CTCTCCGTC GACTCAATTT AAACACCCC
 CAGTAATCAC TGAGTACTTA TAAAAGGAAG GACAGCCACT CTGACTTAAA TTTGATGGG

-2338
 AAGGAACCTT TGGATTCTTT CCTGACAGG AAACAGATTC ACCTTTTTCAG TCGATAGCG
 TTCTTGGAA ACCTAAGAAA CCGACTGTCC TTTGTCTAAC TCGAAAAGTC ACCTATCCCT
*Bam*HI

-2278
 TCGGAGATG
 TCCCTCTAC

Fig. 3.3 DNA sequence of the HD promoter region containing a minimal PPRE. The putative minimal HD PPRE is underlined. The consensus TGACCT half-site for several hormone receptors is shaded. Filled arrows indicate direct repeats of the half-site which later proved to be the the core PPRE; open arrows indicate other TGACCT-like motifs whose functions are unclear.

3.1.3 *Liver-Specific Cellular Factors Interact with the HD PPRE*

To detect the presence of cellular factors that interact with the HD PPRE, mobility shift experiments were performed. Incubation of the radiolabeled probe of the 196-bp fragment containing the minimal HD PPRE (Fig. 3.6, nucleotides -3040 to -2845) with nuclear extracts from H4IIEC3 cells treated or untreated with ciprofibrate resulted in the formation of two major protein-DNA complexes, designated C1 and C2 (Fig. 3.4.A, lanes d and e). Two other minor complexes were occasionally observed under the experimental conditions. When this probe was incubated with nuclear extracts from HeLa or BSC40 cells (lanes b and c), no complexes were observed, suggesting that the DNA-binding factors are liver-specific.

The C1 and C2 complexes were observed with nuclear extracts from both ciprofibrate-treated and untreated H4IIEC3 cells. Interestingly, the C2 complex was more abundant in the treated cells than in the untreated cells (compare lanes d and e). The integrity of each extract was assessed with a DNA probe containing the binding site for a ubiquitous transcription factor, Oct-1 (Werstuck and Capone, 1989). The pattern of the protein-DNA complexes formed with the Oct-1 probe appeared the same for ciprofibrate-treated and untreated H4IIEC3 cells (Fig.3.4.B, lanes b and c). These results suggest that the quantitative and qualitative differences in protein-DNA interactions observed in these cell extracts with the PPRE probe are related to the

A PPRE Probe B Octamer Probe

a b c d e

a b c d

69

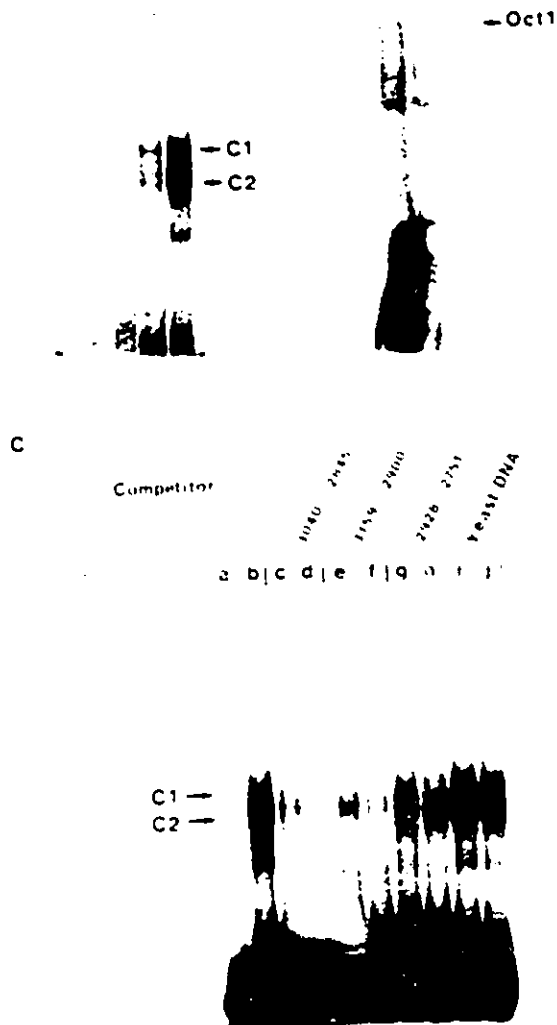


Fig. 3.4 Specific interactions of the HD PPRE with liver cell nuclear factors in mobility shift analysis. (A) Tissue specificity of the HD PPRE binding proteins. The radiolabeled PPRE Probe contains the minimal HD PPRE from nucleotides -3040 to -2845. It was incubated with BSA, or nuclear extracts of HeLa, BSC40, and untreated or ciprofibrate-treated H4IIEC3 cells (lanes a, b, c, d, e, respectively). C1 and C2 indicate the major protein-DNA complexes. (B) Octamer Probe, 5'-GATCCCGTGCATGCTAATGATATTCT-3' contains a binding site for an ubiquitous nuclear protein, *Oct-1*, as underlined (Werstuck and Capone, 1989). It was incubated with nuclear extracts from BSC40, HeLa, untreated or ciprofibrate-treated H4IIEC3 cells (lanes a, b, c, and d, respectively). Lane a was exposed to X-ray film for only 1 hr; other lanes, for 13 hr. (C) DNA sequence specificity of the protein-DNA interactions. Mobility shift analysis was performed with radiolabeled minimal HD PPRE fragment and nuclear extract of ciprofibrate-treated H4IIEC3 cells in the presence of specific competitors: the unlabeled HD PPRE fragment (lanes a and d) or the partially overlapping fragments (lanes e and f; lanes g and h). The numbers indicate the positions of the first and last nucleotides of the competitors. Yeast DNA is a nonhomologous 210 bp fragment from the upstream region of a yeast gene. 50- (lanes c, e, g, and i) or 100- (lanes b, d, f, h, j) molar excess of a competitor was added. No competitor was added for lanes a and b; no nuclear extract for lane a.

treatment with ciprofibrate.

HeLa and BSC40 extracts were also capable of forming the Oct-1 complexes (Fig.3.4.B, lanes a and d), supporting the observation that these cells might not contain specific HD-PPRE binding factors equivalent to those in H4IIEC3 cells. When the mobility shift experiments were repeated with extracts made by an alternative procedure - a detergent-free method, the results were essentially as before (data not shown). Similar quantitative and qualitative differences were again observed with the H4IIEC3 cells due to ciprofibrate treatment. The BSC40 cell extract again showed little binding to the minimal HD PPRE probe (data not shown). The HeLa cell extract made by this method, on the other hand, was detected to contain factors capable of binding to the HD PPRE. These HeLa factors were, however, different from the liver factors in electrophoretic mobility and they reacted with a different antibody (data and details will be further discussed in later chapters). The conclusion on the liver-specificity of the observed HD-PPRE binding complexes in H4IIEC3 cells is, therefore, correct.

Competitive mobility shift experiments were carried out to assess the nucleic acid sequence specificity of the observed protein-DNA interactions. A 50- or 100-fold molar excess of unlabeled probe DNA (the minimal HD PPRE of 196 bp from nucleotides -3040 to -2845) significantly reduced the amounts of both the C1 and C2 complexes (Fig.3.4.C, lanes c and d). In contrast,

nonspecific yeast DNA isolated from the upstream region of a yeast gene (lanes i and j) was unable to compete for complex formation. Thus, liver nuclear factors interact with the HD PPRE in a DNA sequence-specific manner.

Competitor DNA corresponding to nucleotides -3159 to -2900, which overlaps the 5' end of the minimal PPRE probe by 141 nucleotides, blocked the formation of both the C1 and C2 complexes quite effectively (Fig.3.4.C, lanes e and f). A competitor oligonucleotide from -2928 to -2751, which overlaps the 3' end of the minimal HD PPRE probe by 84 nucleotides, was also able to inhibit complex formation, although with less efficiency (lanes g and h). These two competitor oligonucleotides overlap each other by 29 nucleotides, suggesting that this overlapping region may be critical for protein-DNA interactions. This conclusion correlates with the results of the *in vivo* experiments: neither of these two overlapping fragments showed full response to ciprofibrate although the one that shares 141 bp with the 5'-end of the minimal HD PPRE fragment showed a partial response. This analysis points to a close association between liver-factor binding *in vitro* and transactivation *in vivo*.

3.1.4 *Hormone-Responsive Core Motifs are Present in the Minimal HD PPRE*

The HD promoter region up to the *NdeI* site (-3178) was sequenced (Fig. 3.3). A tandem array of three TGACCT motifs was located

between nucleotides -2948 and -2926: TTGACCTATTGAaCTaTtACCTA. The 196 bp of the minimal HD PPRE region covers these TGACCT-like sequences (see also Fig. 3.6). The two smaller fragments (-3159 to -2900 and -2928 to -2751), which were competitive for protein binding to the minimal HD PPRE in mobility shift analysis but were hardly responsive to ciprofibrate *in vivo*, overlapped this TGACCT array. This fact encouraged the prediction that these TGACCT motifs might be critical for both protein-DNA interaction and ciprofibrate induction.

This finding might have been expected. Repeats of the consensus TGACCT motifs have been widely recognized in the hormone-responsive elements (HREs) of a great many genes (Lucas and Granner, 1992). The specific recognition of such an HRE by nuclear hormone receptors is considered the initial signaling event for hormonal regulation of gene expression. Among other things, specificity is defined by the number of repeats, the orientation of the repeats, and the number of spacing nucleotides between the TGACCT motifs (Urbano et al., 1991).

While this project was underway, several groups have showed that peroxisome proliferation can activate nuclear receptors named peroxisome proliferator activated receptors (PPARs), which are homologous proteins of a family of nuclear hormone receptors (Green, 1992; Green et al., 1992). PPARs from mice (Tugwood et al., 1992) and *Xenopus* (Dreyer et al., 1992) are able to transactivate

the expression of the rat peroxisomal acyl-CoA oxidase (AOx) gene, which encodes the first enzyme of the peroxisomal β -oxidation pathway (Osumi et al., 1991). The PPRE in the AOx promoter contains two TGACCT motifs tandemly arranged with one nucleotide in between. Data presented in this section suggested some similarities between the HD PPRE and the AOx PPRE. Their functions are independent of orientation and position relative to the transcription start site. Both PPREs confer responsiveness onto a heterologous promoter. Both interact with liver-specific factors in a DNA sequence-specific manner.

In contrast to the AOx PPRE, the HD PPRE is located more than 2 kbp farther upstream from its transcription start site. Secondly, the HD PPRE is flanked by unique sequences, some of which have also been associated with hormonal gene regulation. As discussed earlier, the construct i was insufficient for maximal induction, although it included the complete sequence of the TGACCT direct repeats and it did show some binding to the same TGACCT-binding proteins in a mobility shift competition. The sequence downstream from the TGACCT direct repeats was also a weak competitor for protein binding (Fig. 3.4, lane g and h) but was unresponsive to ciprofibrate (Fig. 3.2, plasmid j). It can be, therefore, assumed that the TGACCT direct repeats in the HD PPRE is required for induction but a proper coordination of several flanking sites are required for a full response. It will be very interesting to determine the nature of the protein-DNA interactions on the minimal

HD PPRE and the possible interplay of ligand-activated receptors, both in protein-DNA complex formation and in coordinated transcriptional activation.

3.2 Interactions between the HD PPRE and Rat Liver Nuclear Proteins

3.2.1 *Interactions of Rat Liver Nuclear Factors with the Minimal HD PPRE*

The 196-bp fragment of the minimal HD PPRE (-3040 to -2845) contains a tandem array of TGACCT motifs. In many hormone responsive elements, the motif serves as a half binding site for tissue specific transcription factors. In this minimal HD PPRE, these motifs may constitute a core element mediating the observed protein-DNA interactions and the ciprofibrate-responsiveness.

In order to define further this and other elements in the minimal HD PPRE fragment with respect to its interactions with nuclear factors, DNase I footprint analysis was performed using nuclear extracts from rat H4IIEC3 cells. The footprint pattern revealed two regions protected from DNase I digestion (Fig. 3.5). On the upper strand, region I covers the 5'-end of the probe up to -3014, while region II spans approximately nucleotide -2985 to -2927. In addition, a DNase I-hypersensitive site that is dependent on protein binding is observed at approximately nucleotide -2964 in the lower strand (Fig. 3.5 and 3.6, arrowhead).

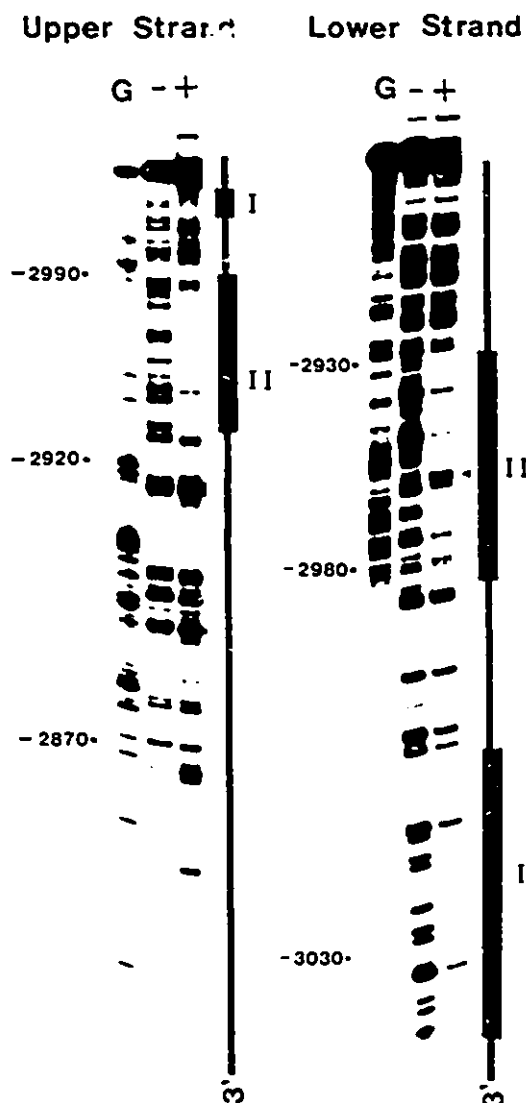


Fig. 3.5 DNase I footprint analysis of the HD PPRE. The 196 bp of the minimal HD PPRE fragment (-3040 to -2845) was radiolabeled at its 3'-end of either the *Upper* or the *Lower Strand* and was subjected to DNase I footprint analysis in the presence of nuclear extract of H4IIEC3 cells (+) or with a BSA control (-). The *numbers* represent the nucleotide positions which are based on a G reaction of chemical sequencing (G). The *dark boxes* correspond to the protected regions I and II. The *arrowhead* points to a site hypersensitive to DNase I.

Both protected regions contain multiple repeats of the common TGACCT-like motifs that are required for steroid hormone receptor signaling (Kliwer et al., 1992b). Region I contains three such motifs. The first motif is followed by two inverted repeats separated by four and two nucleotides, respectively (Fig. 3.6, TGACCActagATGTCaggAGGTAA, nucleotides -3037 to -3014, in highlighted region I). Region II also contains three repeats of the TGACCT motif. They are, however, present in a directly repeated arrangement and are separated by two nucleotides and one nucleotide, respectively (TGACCTatTGAACtATTACCT, nucleotides -2947 to -2927, in highlighted region II). The protection in region II extends upstream from the TGACCT array to include two directly repeated CACCC boxes (Fig. 3.5, nucleotides in bold italics) and several additional imperfect CACCC motifs between nucleotides -2980 and -2954. CACCC boxes have been shown to be important for hormone receptor-mediated responsiveness of several genes whose responsive elements are, like HD PPRE, far upstream of their transcription start sites (Strähle et al., 1988). The DNase I hypersensitive site, which is considered an indicator of the boundary of a protein-DNA complex, is positioned between the TGACCT-like direct repeats and CACCC motifs, indicating the possible presence of more than one protein complex in region II.

The second and third TGACCT motifs in the protected region II resemble the minimal AOx PPRE, which contains two directly repeated TGACCT-like motifs separated by a single nucleotide, TGACCTtTGTCCT,

-3040 **I**

CTGTGACCACTAGATGTCAGGAGGTAAAGTGTGAGTCTGCCTCTGTTTTTCTCTGCTCCC
GACACTGGTGATCEACAGTCCCTCCATTCACACTCAGACGGAGACAAAAAGAGACGAGGG

-2980 **II**

CACCCACCCACCGCTACCCACCTCTCCTTTGACCTATGAACTATTACCTACATTT
GTGGGGTGGGGTGGCGATGGGGGTGGAGAGGAACTGGATAACTTGATAATGGATGTAAA

-2920

GAGTGCCCGAGACTTTGGGAGCTGATAGGCTTAAACATTTGCTGAACGAGTAAAAAG
CTCACGGGGCTCTGAAACCCTCGACTATCCGAAATTGTAAACGACTTGCTCATTTTTC

-2860 -2845

TAAATAAATTCAGGA
ATTATTTAAAGTCCT

Fig. 3.6 DNA sequence of the minimal HD PPRE. The numbers correspond to nucleotide positions with respect to the transcription start site. The regions protected from DNase I digestion are enclosed by lines. The arrow indicates the hypersensitive site. The shaded sequences are variations of the consensus TGACCT motif found in many HREs. The italics are CACCC boxes found in the vicinity of several HREs.

corresponding to nucleotides from -570 to -558 upstream of the transcription start site (Issemann and Green, 1990). The AOx PPRE has been shown to interact with the mouse PPAR and thus to mediate the peroxisome proliferator-induced transactivation (Tugwood et al., 1992). Because of its similarity to the directly repeated core motifs of the AOx PPRE, the sequence containing the TGACCT direct repeats in the HD PPRE was chosen to be further characterized and compared to the AOx PPRE with respect to nuclear factor binding and peroxisome proliferator-responsiveness.

Double-stranded oligonucleotides corresponding to nucleotides -2956 to -2919 of the HD PPRE or to the AOx minimal PPRE (nucleotides -583 to -544) were synthesized and radiolabeled for mobility shift assays with H4IIEC3 nuclear extracts (Fig. 3.7). In a control experiment, the 196-bp fragment of the minimal HD PPRE was shifted to a major protein/DNA complex when incubated with H4IIEC3 nuclear extract (lanes labeled HD-PPRE). A major protein/DNA complex was also formed with both the HD (lanes labeled HD) and AOx oligonucleotides (lanes labeled AOx). The respective complexes with the two oligonucleotides showed comparable electrophoretic mobility. Since the HD and AOx oligonucleotides were of similar length (41 and 44 bp, respectively), these results suggest that similar nuclear factors interact with both the HD and AOx elements.

This similarity is shown more directly by competition analysis

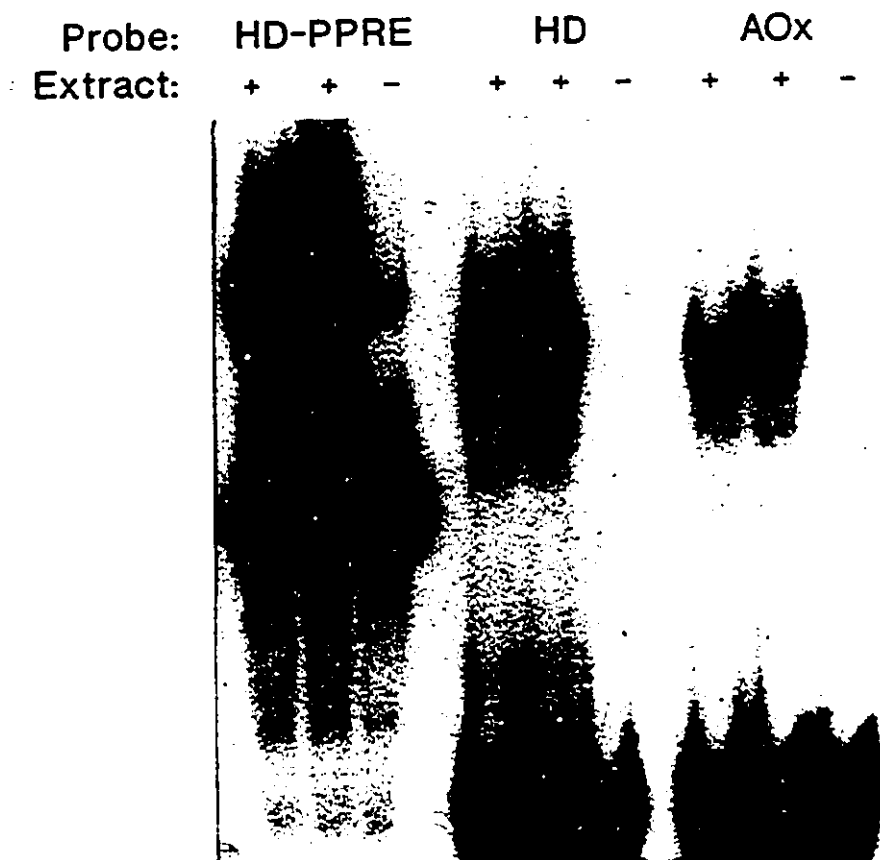


Fig. 3.7 H4IIEC3 nuclear factors interact with the HD and AOx PPRE-containing oligonucleotides in mobility shift assays. *HD-PPRE* is the entire 196 bp of the minimal HD PPRE fragment from -3040 to -2845 used in DNase I footprint analysis. *HD* is a synthetic oligonucleotide from -2956 to -2919 which contains three directly repeated TGACCT-like motifs. *AOx* contains the AOx PPRE from the AOx promoter, from nucleotides -583 to -544. Incubation was carried out with (+) or without (-) nuclear extracts from H4IIEC3 cells. The nuclear extracts were prepared from cells treated or not treated with ciprofibrate (every first and second lane, respectively). The band in the control lane (-) under HD-PPRE is a result of spillover from the adjacent lane.

(Fig. 3.8). Both the AOx and HD oligonucleotides were capable of competing for factor binding by the 196-bp minimal HD PPRE fragment (top panel), although not as effectively as the minimal HD PPRE itself. Reciprocally, the unlabeled minimal HD PPRE fragment prevented factor binding to both the radiolabeled HD and AOx oligonucleotides. In fact, this minimal HD PPRE fragment was a more effective competitor than the corresponding unlabeled oligonucleotides as self-competitors (middle and lower panels, respectively).

The unlabeled AOx oligonucleotide competed for factor binding by the radiolabeled HD oligonucleotide, but it was not as effective as the unlabeled HD oligonucleotide as a self-competitor (middle panel). *Vice versa*, the unlabeled HD oligonucleotide competed less effectively than the AOx oligonucleotide as a self-competitor for binding to the AOx element (bottom panel). These results were confirmed when the competitors were added as 3, 6, 12.5, 25, 50, 100, and 200 molar excess of a radiolabeled probe (data not shown). In all these experiments, non-specific DNA (NS) as a competitor had no effect on protein binding, indicating that the observed liver factor recognition is DNA sequence specific. Taken together, the same or similar nuclear factors from rat hepatoma H4IIEC3 cells can recognize both the AOx and HD elements and that the sequence encompassing the three TGACCT direct repeats in the HD element is a major element for liver factor binding to the HD PPRE. Since little difference was observed at this point between these

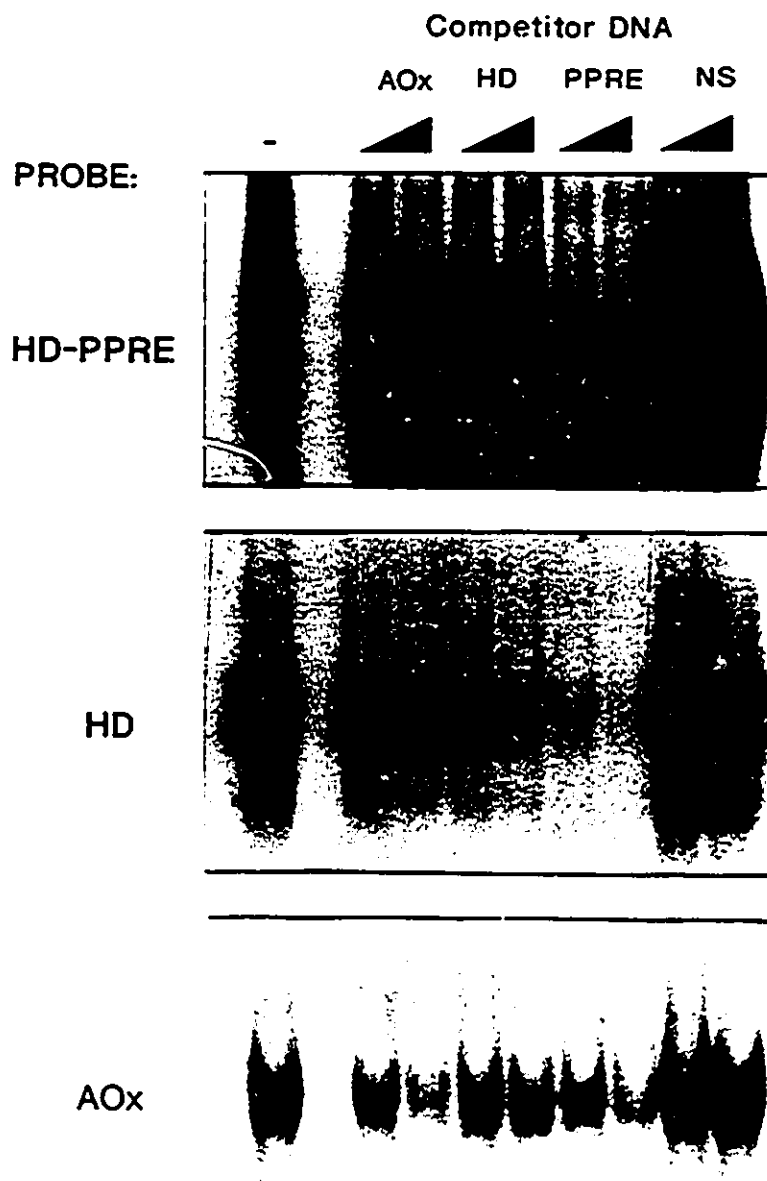


Fig. 3.8 Similar nuclear factors bind to both the HD and AOx PPRE-containing oligonucleotides. The *PROBES* were radiolabeled oligonucleotides containing the HD or AOx element. *HD-PPRE* is the 196 bp minimal HD PPRE fragment. The specific *Competitor DNAs* are unlabeled DNA fragments used as probes, and *PPRE* is the same fragment as the probe *HD-PPRE*. The non-specific competitor (*NS*) is a 210 bp fragment from yeast DNA. Approximately 50- and 100- fold molar excess of competitor was added (every first and second lane, respectively, for each competitor, *solid triangle*). The reaction on the far left lane of each panel was performed without competitor (-). Free probes are not shown.

ciprofibrate-treated and untreated nuclear extracts, only the drug-treated extracts were used hereafter.

3.2.2 *Factor Binding Sites within the HD TGACCT Motifs*

Whether specific nucleotides within the sequence of the core HD PPRE were important for liver factor binding was investigated by methylation interference analysis using H4IIEC3 nuclear extracts and the HD oligonucleotide corresponding to nucleotides -2956 to -2919 (Fig. 3.9.A). Methylation of the G residue at position -2935 on the lower strand remarkably disrupted protein binding (dark dot), while methylation of the two Gs at -2929 and -2928 on the lower strand only partially interfered with binding. The first TGACCT core motif from the 5'-end of the three repeats does not appear to be important for protein binding, since methylation of the Gs on either strand within this motif had little effect on the observed protein/DNA interaction.

For comparison, the methylation interference pattern of the AOx element was analyzed in parallel using the AOx oligonucleotide (Fig. 3.9.B). Methylation of the G residue at position -569 or the Gs at positions -571 and -562 on the upper strand either completely or partially interfered with factor binding. Methylation of the Gs at -567 and -566 and at the symmetrically disposed Gs at -560 and -559 of the downstream TGACCT half-site on the lower strand also prevented binding. In contrast, the Gs at -2935, -2929, and -2928 in the lower strand of the HD element that partially interfered

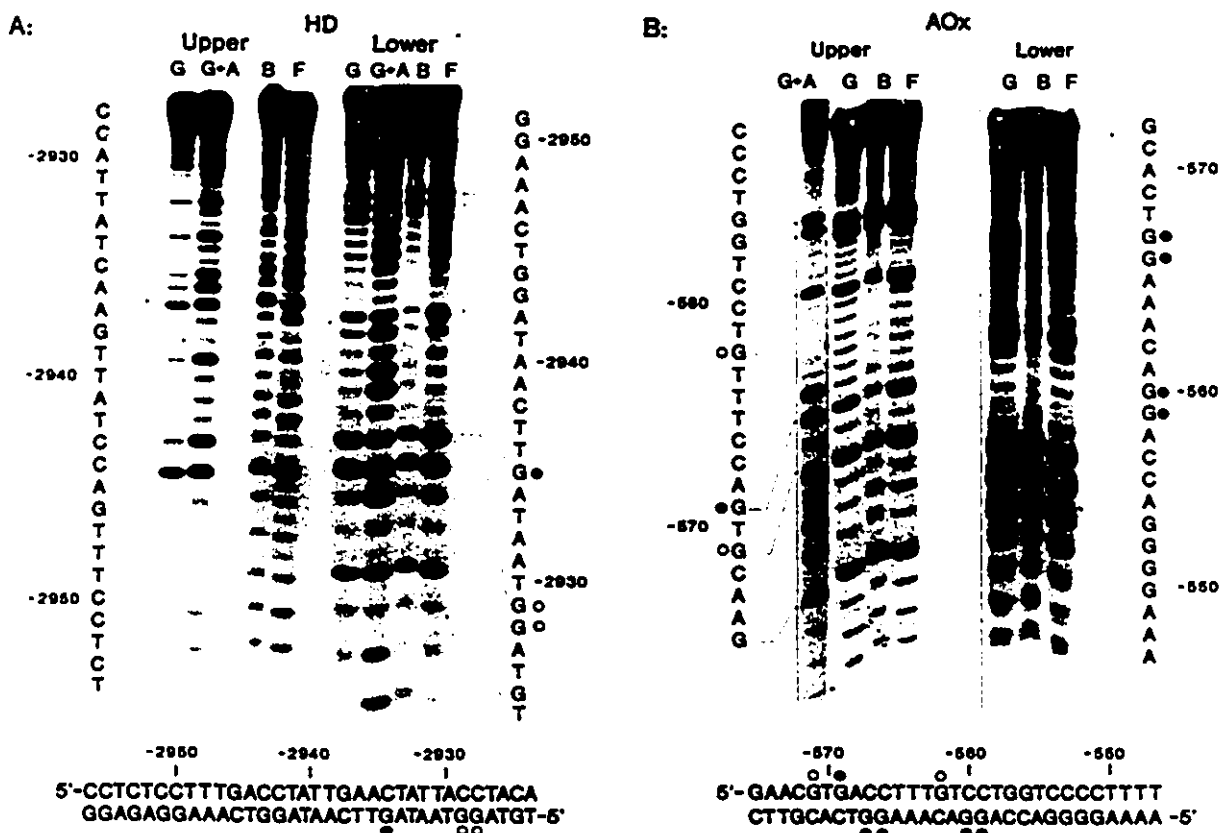


Fig. 3.9 Methylation interference analysis of the HD and AOx elements. Oligonucleotides of the HD PPRE (panel A) and AOx PPRE (panel B) were radiolabeled at the 5'-ends of either the *Upper* or *Lower* strand, methylated with dimethyl sulfate, and incubated with H4IIEC3 nuclear extract. The bound (*B*) and free (*F*) probes were isolated by mobility shift assay, and then cleaved with piperidine. Methylation of certain G residues is shown to partially (open circles) or strongly (closed circles) interfere with the binding of liver factors to the PPREs. The nucleotide sequences are shown on the sides and bottom. The numbers indicate the positions of the nucleotides which are based on G and G+A reactions of DNA chemical sequencing.

with factor binding correspond in position to the Gs at -566, -560, and -559 on the lower strand of the AOx element, which were also shown to be sensitive to methylation. It appears, therefore, that the two downstream core motifs of the HD element are similar to the two core motifs in the AOx element and are important for the specific factor binding observed in mobility shift analysis. This conclusion is consistent with the fact that the spacing between these two motifs of the HD element is the same as that separating the two AOx motifs.

Some important differences were revealed in the interference patterns of the HD and AOx elements. For instance, methylation of the G at -2938 of the upper strand of the HD element had no effect on factor binding, while methylation of the corresponding G at position -569 of the upper strand of the AOx element completely prevented binding. Overall, the data indicate that, although the same or similar nuclear factors interact with the HD and AOx elements, they do so in different ways. Alternatively, there may be different components in the protein complexes on the two PPREs. This was shown to be true in later experiments.

To investigate further the role of specific nucleotides in the HD element in protein binding, selected mutations were introduced into the TGACCT motifs in the HD FPPE. The mutant oligonucleotides were used in mobility shift assays (Fig. 3.10). Mutation of the G residues to Cs in the upper strand of the first and second TGACCT-

like half sites at positions -2946 and -2938 (M1) reduced markedly, but did not eliminate, factor binding (panel A, compare lane e to lane a). Additional changes on the M1 to convert the Cs to Gs at positions -2944, -2943, and -2935 (M2) completely prevented factor binding (panel A, lane c). Consistently, in the competition analysis (panel B), the unlabeled M1 was able to compete for factor binding to the wild type HD PPRE probe, although it was not as effective as the unlabeled wild type HD oligonucleotide itself (panel B, compare lanes M1 and HD). As had been expected, the M2 oligonucleotide, which was completely defective in factor binding (see panel A, lane c), was unable to compete with the wild type HD PPRE probe (panel B, lanes under M2).

Since methylation of a single G residue at position -2935 of the lower strand of the HD oligonucleotide significantly interfered with factor binding (Fig. 3.9.A), an M3 oligonucleotide was designed with a G→C transversion at this position. In agreement with the methylation interference data, mutation of this single base pair significantly reduced protein binding to the wild type HD oligonucleotide probe (Fig. 3.11, compare lanes c and a). In summary, these mutation analyses support the finding that the second, and probably also the third, TGACCT-like motifs in the HD element are the critical determinants for liver factor binding.

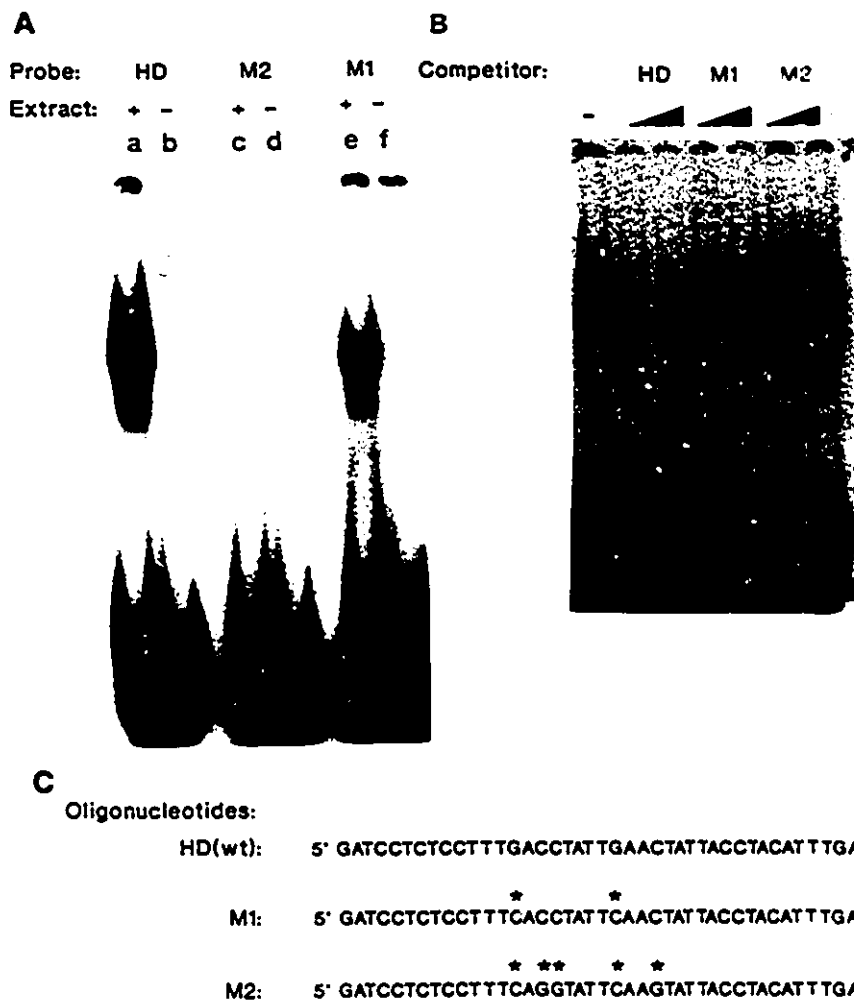


Fig. 3.10 Effects of mutations in the HD PPRE on nuclear factor binding in mobility shift assays. (A) Oligonucleotides of the HD PPRE (-2956 to -2919) and its mutants M1 (with two Gs in the lower strand mutated) and M2 (with the two Gs in the lower strand and 3 Gs in the upper strand mutated) were radiolabeled and incubated with (+) or without (-) nuclear extracts of H4IIEC3 cells. (B) The mobility shift assay was performed in the presence of competitors: 50- or 100- fold molar excess (solid triangles) of unlabeled oligonucleotides of the wild type HD PPRE, or the HD mutants M1 or M2. No competitor was added in the control reactions (-). (C) Nucleotide sequences of the probes. The stars indicate the mutated nucleotides.

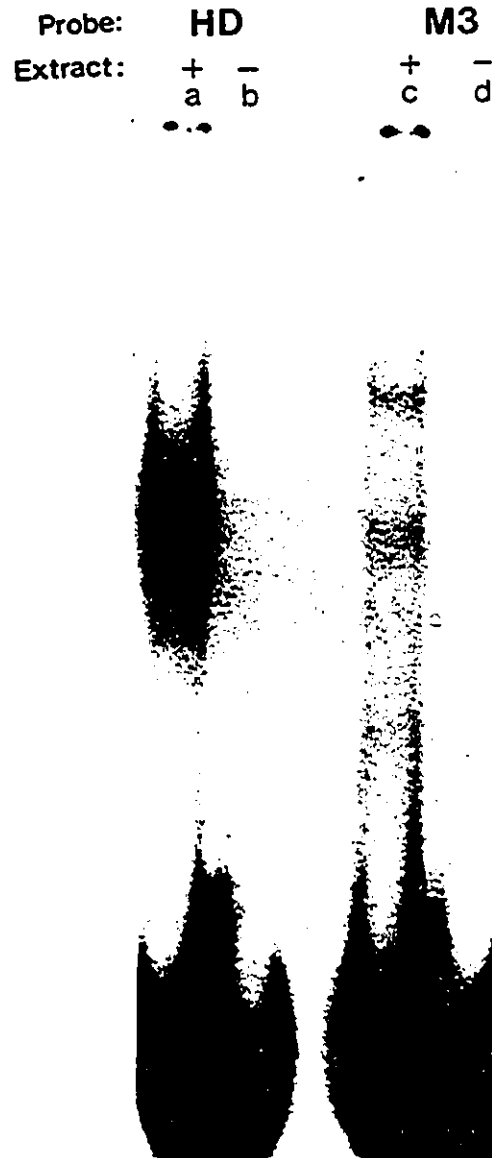


Fig. 3.11 Mutation of one G-residue in the core HD PPRE abolishes binding of H4IIEC3 nuclear factors to the element. The oligonucleotide of the wild type HD PPRE and its mutant (M3) with one G converted to C (see Materials and Methods for sequence) were radiolabeled and incubated with (+) or without (-) nuclear extract of H4IIEC3 cells.

3.2.3 *The Oligonucleotide HD Element is Sufficient to Confer Peroxisome Proliferator-Responsiveness onto a Heterologous Promoter*

The 196-bp fragment of the minimal HD PPRE was able to confer full ciprofibrate-responsiveness when linked to the unresponsive CPS basal promoter following transfection of H4IIEC3 cells. In order to determine if the three TGACCT direct repeats in the minimal HD PPRE are by themselves sufficient to confer ciprofibrate-responsiveness, an oligonucleotide containing the three TGACCT direct repeats was linked to the 5'-end of the CPS promoter in pCPS_{luc}. It was inserted into the vector either as a single copy or three tandemly ligated copies.

These plasmids were transiently transfected into H4IIEC3 cells in the presence or absence of ciprofibrate. Luciferase activity in the cell lysate was measured (Fig. 3.12). As a control, the 196 bp of the minimal HD PPRE (nucleotides -3040 to -2845; pHD-PPRE_{luc}) resulted in an approximately 14-fold induction of luciferase activity in the presence of ciprofibrate. The luciferase activity of pHD(X1)_{luc}, which contains one copy of the HD oligonucleotide, was induced ~3.5-fold, while the activity of a plasmid containing three tandem copies of the HD oligonucleotide, pHD(X3)_{luc}, was induced 10-fold by ciprofibrate. These results demonstrate that the HD oligonucleotide sequence encompassing the TGACCT direct repeats is not only necessary but also sufficient to confer ciprofibrate-responsiveness to a normally unresponsive heterologous promoter and that the level of induction can be increased by multimerization of

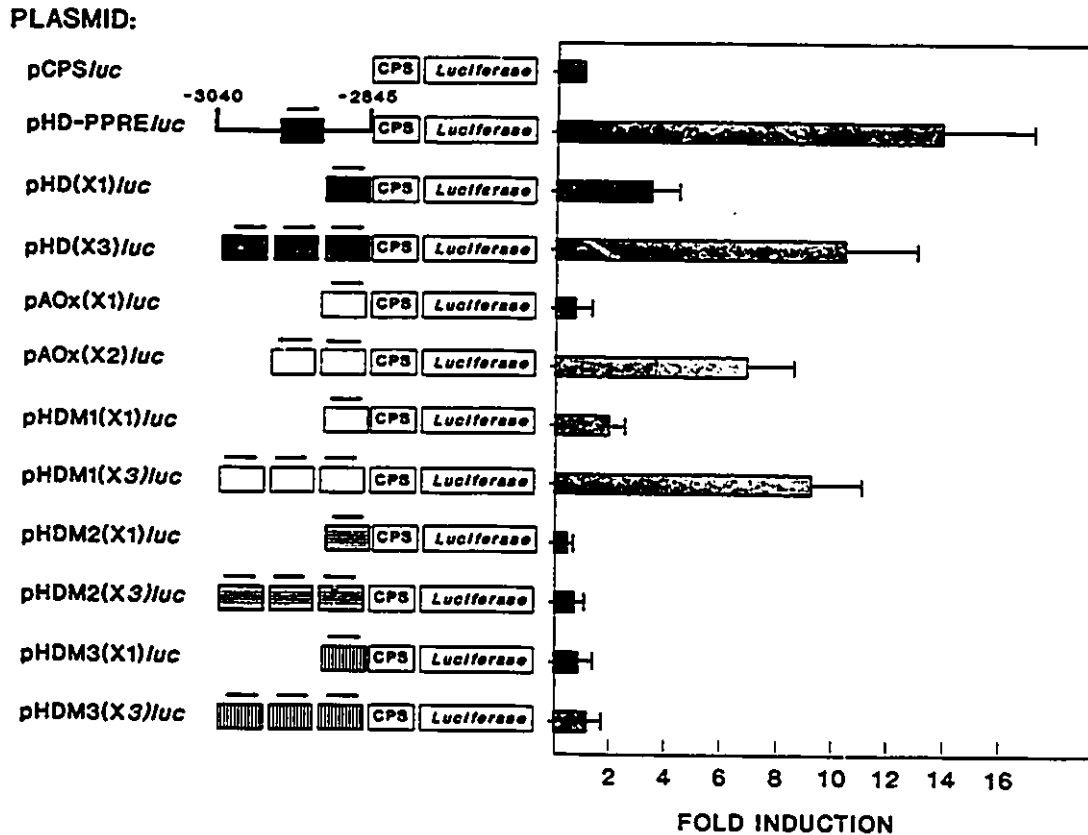


Fig. 3.12 . Ciprofibrate-responsiveness is mediated by the core HD element and requires the binding of nuclear factors. H4IIEC3 cells were transfected with the pCPSluc-derived plasmids carrying one (X1) or three (X3) copies of the oligonucleotides containing the core HD PPRE (-2956 to -2919) or its mutants (M1, M2, M3, see Materials and Methods for sequences). Also, the plasmids containing one (X1) or two (X2) copies of the AOx PPRE oligonucleotide, a plasmid with the 196 bp of the minimal HD PPRE (-3040 to -2845), and the empty vector pCPSluc were tested. The arrows represent the orientations of the inserts. The fold induction is the value of the luciferase activity in the presence of ciprofibrate over that in the absence of drug and is normalized to the result of the pCPSluc control in the absence of drug. The values are averaged from at least three independent transfections, each being carried out in duplicate. The average induction ratio of pCPSluc is 0.5 ± 0.03 . The absolute luciferase activity ranges from 1 to 3 integrated light units.

the element. Therefore, this element containing the three TGACCT direct repeats is the core HD PPRE.

3.2.4 *Factor Binding to the TGACCT Direct Repeats is Required for Ciprofibrate-Mediated Induction*

In order to determine if the binding of nuclear factors to the HD element is necessary for ciprofibrate-mediated induction of gene expression, single and multiple copies of the HD mutant oligonucleotides (M1, M2, and M3) were inserted into pCPS1uc. The constructs were assayed for inducibility by ciprofibrate in H4IIEC3 cells (Fig. 3.12).

The luciferase activity of pHDM1(X1)luc was weakly induced by ciprofibrate to only ~ 2-fold; however, a plasmid containing three copies of the M1 oligonucleotide in tandem arrangement, pHDM1(X3)luc, was induced ~ 9.5-fold. In contrast, plasmids containing single or multiple copies of the M2 or M3 oligonucleotides (with mutations of five Gs in both strands or one G in the lower strand of the second TGACCT motif, respectively) were unresponsive to ciprofibrate. These observations are consistent with the results from mobility shift analysis that the M1 oligonucleotide interacted with liver nuclear factors (Fig. 3.10 and 3.11), whereas the M2 and M3 oligonucleotides failed to do so. In addition, the density of the shifted complex with M1 was consistently lower than the complex on the wild type HD probe (Fig. 3.10), which was reflected quantitatively in the results from

transfections of the pHD(X1)luc and pM1(X1)luc (Fig. 3.12). Thus, recognition of the TGACCT repeats in the core HD PPRE by nuclear transcription factors is required for peroxisome proliferator-mediated induction of HD gene expression.

Therefore, the sequence with the three TGACCT-like direct repeats corresponding to nucleotides -2956 to -2919 in the HD promoter acts as the core element of the HD PPRE. As demonstrated by DNA binding, competition, and methylation interference analyses, the same, or similar, rat liver nuclear factors bind to the HD element and the AOx PPRE, which consists of two TGACCT motifs. Mutations in the HD element that abolish factor binding *in vitro* also preclude the mutant from responding to ciprofibrate *in vivo*, directly demonstrating that binding of nuclear transcription factors to the PPRE is a prerequisite for peroxisome proliferator signaling.

3.2.5 *Auxiliary Elements in the Minimal HD PPRE Bind to Liver Nuclear Factors*

It is conclusive that the sequence with the three TGACCT direct repeats renders a threshold responsiveness to ciprofibrate. However, the induction ratio from transfection of either pHD(X1)luc or pHD(X3)luc was reproducibly less than that observed with the 196-bp of the minimal HD PPRE. Thus, whereas the TGACCT direct repeats are necessary and sufficient for ciprofibrate induction, flanking sequences may contribute to full responsiveness.

To further explore these flanking sequences, DNase I footprint analysis of the 196-bp probe of the minimal HD PPRE was performed in the presence of the unlabeled HD oligonucleotide as a competitor. The HD oligonucleotide contains the three TGACCT direct repeats with little flanking region (Fig. 3.13). The oligonucleotide containing the TGACCT direct repeats efficiently competed for the protected region I in a concentration-dependent fashion. In the protected region II, on the other hand, the TGACCT-containing oligonucleotide only deprotected its own sequence. It had hardly any effect on the protection of the rest of the region, which contained several CACCC boxes (for the DNA sequence, see Fig. 3.6). This suggests that there exist at least two independent complexes in region II. One is based on the TGACCT direct repeats, and the other shows dependence on the CACCC boxes for binding. The hypersensitive cleavage site (arrowhead) probably indicates the boundary of the two protein binding sites.

The complete inducible HD enhancer, therefore, includes the TGACCT direct repeats, as well as flanking elements. The concept of a complete promoter as part of a hormone response unit has been reviewed in Lucas and Granner (1992). Briefly, extensive evidence has shown that multiple short *cis* elements exist in the promoter region of an inducible gene, each being a simple binding site for a cognate transcription factor. It is the unique combination of these simple elements in a given gene that determines the magnitude, direction, and specificity of the hormonal

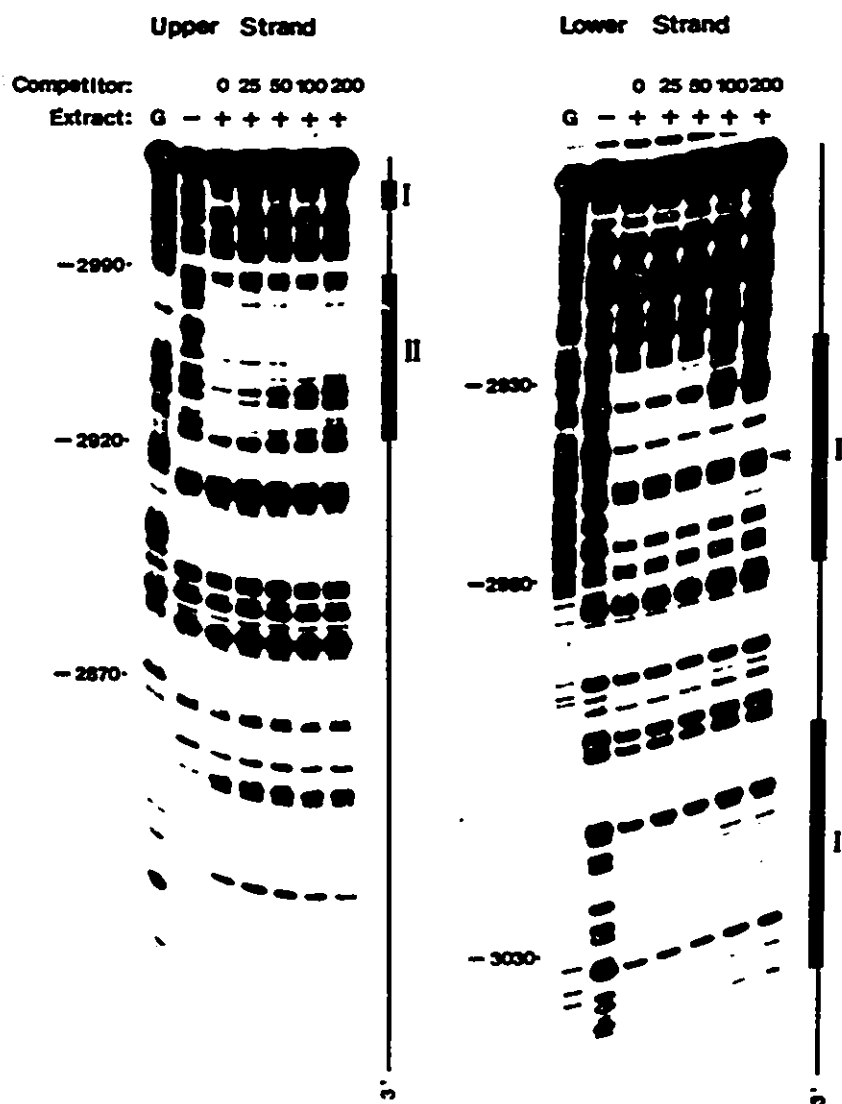


Fig. 3.13 In the DNase I-protected region I, TGACCT motifs and CACCC boxes are protected by two different protein complexes. The 196 bp fragment of the minimal HD PPRE (-3040 to -2845) was radiolabeled at the 3'-ends of either the *Upper* or *Lower Strand* and was subjected to DNase I digestion with (+) or without (-) nuclear extracts of H4IIEC3 cells. 0-, 25-, 50-, 100-, or 200-fold molar excess of a competitor, the oligonucleotide containing the three TGACCT direct repeats (-2956 to -2919), was added. The numbers are the nucleotide positions, which are determined by the G reaction of the chemical sequencing of the probe (G). The dark boxes correspond to the protected regions I and II. The arrowhead points to a DNase I-hypersensitive site.

responsiveness of that gene. The complete HD PPRE, therefore, includes two groups of TGACCT-like half sites and one group of CACCC motifs.

Although the function of the CACCC box has not been extensively explored, this promoter sequence signature has been found in many liver specific genes (Schüle et al., 1988). When placed in the immediate vicinity of a GRE in the promoter region of the thymidine kinase gene, it facilitated the maximal enhancement of the response of the gene to glucocorticoids. In another case (Myers et al., 1986), removal of a CACCC box from the β -globin promoter abolished glucocorticoid induction. A CACCC-binding protein has been mentioned by Schültz's group (Strähle et al., 1988). Their studies also showed that the CACCC box could experimentally replace other auxiliary HRE sequences such as a CCAAT motif, the binding site for the transcription factor SP1, predicting a possible functional homology between the CACCC protein binding site and other nuclear protein binding sites in mediating the regulation of several genes (Park et al., 1993; Umek et al., 1991). The role of the CACCC boxes in the regulation of the HD gene remains to be investigated.

The second auxiliary sequence located further upstream also consists of TGACCT-like motifs, two symmetrically disposed motifs followed by a third separated by four and two nucleotides, respectively. The fact that this protein-binding element can be competed out completely by the 38 bp of the HD oligonucleotide

containing three direct repeats of TGACCT suggests that similar proteins are involved in both binding sites. However, only the elements of direct repeat were sufficient to confer ciprofibrate-responsiveness in H4IIEC3 cells. Therefore, only the sequence of TGACCT direct repeats is considered a true positive PPRE, while the function of the other TGACCT element in relation to peroxisome proliferator signaling is unclear at this point. It is possible that it could mediate a different signaling pathway. It has been reported that RAR and TR bind to direct or inverted repeats, whereas glucocorticoid receptor (GR) and estrogen receptor (ER) bind exclusively to inverted repeats (Katashira *et al.*, 1992). The spacers of 3, 4, or 5 nucleotides confer a specific response to VD₃R, TR, and RAR, respectively (Näär *et al.*, 1989; Umesono and Evans, 1989; Umesono *et al.*, 1990). Considering that several natural hormonal factors possess properties of the peroxisome proliferators, it would not be surprising that these TGACCT-like motifs in the protected region I could mediate response to different signals.

The AOx promoter was also revealed by DNase I footprint analysis to contain two closely located protein-binding sites (Osumi *et al.*, 1991). Site A has been well defined as a positive AOx PPRE by several laboratories, including ours. It consists of two directly repeated TGACCT motifs with one nucleotide spacing (DR1). The protein binding site B downstream of this positive AOx PPRE resembles the half binding site for nuclear transcription factors

ENF1. In transactivation assays, site B showed a negative regulatory activity (Osumi et al., 1991), and did not respond to the three *Xenopus* PPARs ($xPPAR\alpha$, β , and γ ; Dreyer et al., 1992). Thus, whereas the TGACCT direct repeats are core elements for ciprofibrate induction, both the HD PPRE and the AOx PPRE are composed of multiple *cis* elements, which are themselves simple short sequence signatures acting as protein binding sites. It is the unique combination of these protein binding sites in number, space, and orientation that defines the specificity of these genes (Schibler and Sierra, 1987).

3.3 PPAR/RXR α Dimer Mediates Ciprofibrate Induction through the TGACCT Direct Repeats in the HD PPRE.

3.3.1 PPAR and RXR α are Obligatory for Ciprofibrate-Induction

While this project was underway, several groups identified two subtypes of nuclear proteins: PPAR and RXR α , which dimerize on the AOx PPRE and transactivate a downstream reporter gene (Kliwer et al., 1992a; Gearing et al., 1993). PPARs from different species belong to the superfamily of nuclear transcription factors (Dreyer et al., 1993; Issemann and Green, 1990; Gearing et al., 1993; Schmidt et al., 1993). They recognize the consensus TGACCT half site through a distinct P box (CEGCKG) in these proteins. Their ligands are yet to be identified (Issemann and Green, 1990). RXR α is another member of the family of nuclear transcription factors and is the receptor for 9-*cis* retinoic acid (Wolf and Phil, 1993;

Mangelsdorf, 1992; Gearing et al., 1993; Kliewer et al., 1992b).

Our laboratory has demonstrated that exogenous PPAR and RXR α from different species can dimerize on the HD PPRE and transactivate a reporter gene as found for the AOX PPRE (Marcus et al., 1993). Gearing et al. (1993) showed similar results in a co-transfection system with an AOX PPRE-containing plasmid and cDNA plasmids expressing the rat PPAR and RXR α . In all these studies, PPAR and RXR α cooperatively bind to the PPRES, presumably as heterodimers, but neither receptor alone is capable of binding to a PPRE. Thus, mobility shift analysis and co-transfections of a PPRE-driven reporter plasmid with PPAR- and RXR α -expression plasmids have together confirmed that PPAR/RXR α cooperativity and binding to a PPRE are obligatory for peroxisome proliferator-dependent transcription activation.

The physiological significance of a PPAR-RXR α dimer is that both receptors are most highly expressed in the liver and kidneys, the principal target tissues of peroxisome proliferators (Kliewer et al., 1992a). PPAR/RXR α -mediated regulation of the peroxisomal β -oxidation genes is a function of RXR α in overall lipid metabolism. RXR α mediates the response to signals of lipid homeostasis such as fatty acids and retinoic acids (Keller et al., 1993; Dreyer et al., 1993). It ultimately regulates, as a co-factor, target genes such as those encoding fatty acid binding proteins, microsomal fatty acid ω -oxidation enzymes, and lipid transport proteins (Rottman et

al., 1991; Muerhoeff et al., 1992; Wolf and Phil, 1993). The convergence of retinoic acid and peroxisome proliferator signaling pathways has, therefore, wide biochemical significance, because RXR α is a common messenger for many hormonal factors through protein-protein interactions between RXR α and other hormone nuclear receptors such as TR, RAR, VD₃R, or RXR α itself (Leid et al., 1992). Hormone-responsive elements (HREs) regulated by RXR α consist of direct repeats of TGACCT (Leid et al., 1992). The core element in the HD PPRE isolated here is, therefore, a typical target site for RXR α -mediated partnership.

3.3.2 *Presence of Endogenous PPAR and RXR α in the Complexes of Rat Liver Factors on the HD PPRE*

The methylation interference pattern with rat liver nuclear factors on the AOx PPRE presented earlier in this thesis (Fig. 3.9.B) is similar to published data using purified mouse PPAR (Tugwood et al., 1992). This suggests that the nuclear factors from H4IIEC3 cells interacting with the AOx PPRE and the HD PPRE *in vivo* include an endogenous rat PPAR.

To test this hypothesis, a mobility shift assay using H4IIEC3 cell nuclear extracts and the core HD PPRE probe was performed in the absence or presence of polyclonal antibodies against hRXR α (Fig. 3.14, lanes b and b') or mPPAR (lanes c and c'). Supershifted species were formed in the presence of antibodies, showing that endogenous RXR α and PPAR in rat hepatoma cells were indeed involved

in the formation of the protein-PPRE complex. The same experiment with the AOx PPRE probe resulted in similar supershifted species (Fig. 3.14, left panel). The results support the conclusion that transcriptional induction of the HD and AOx gene by peroxisome proliferators is coordinated by the same or similar ligand-activated nuclear receptors, including PPAR- and RXR α -related proteins.

3.3.3 *The DR1 Structure in the Core HD PPRE Mediates the Cooperative Binding of Rat PPAR and RXR α In Vitro*

A dimer of nuclear factors often targets a pair of half sites, as in the case of the AOx PPRE. The three TGACCT direct repeats in the core HD PPRE could thus provide two potential overlapping targets for dimers: the first and second direct repeats are separated by two nucleotides (DR2); the second and third repeats are separated by one nucleotide (DR1). It has been clearly established that the spacing between the TGACCT motifs plays a critical role in achieving selective hormonal response (Umesono and Evans, 1989). It was, therefore, proposed that the DR1 is the major site to accommodate the PPAR/RXR α dimer. Mutation of the first TGACCT half site (M4) had little effect on PPAR- and RXR α -mediated protein binding onto the HD PPRE, as indicated by the identical supershift pattern with the mutant probe and the wild type HD PPRE and AOx PPRE probes (Fig. 3.14). It is, therefore, the DR1 arrangement that provides the binding site for the PPAR/RXR α dimer. In later chapters, this conclusion is supported by similar mobility shift

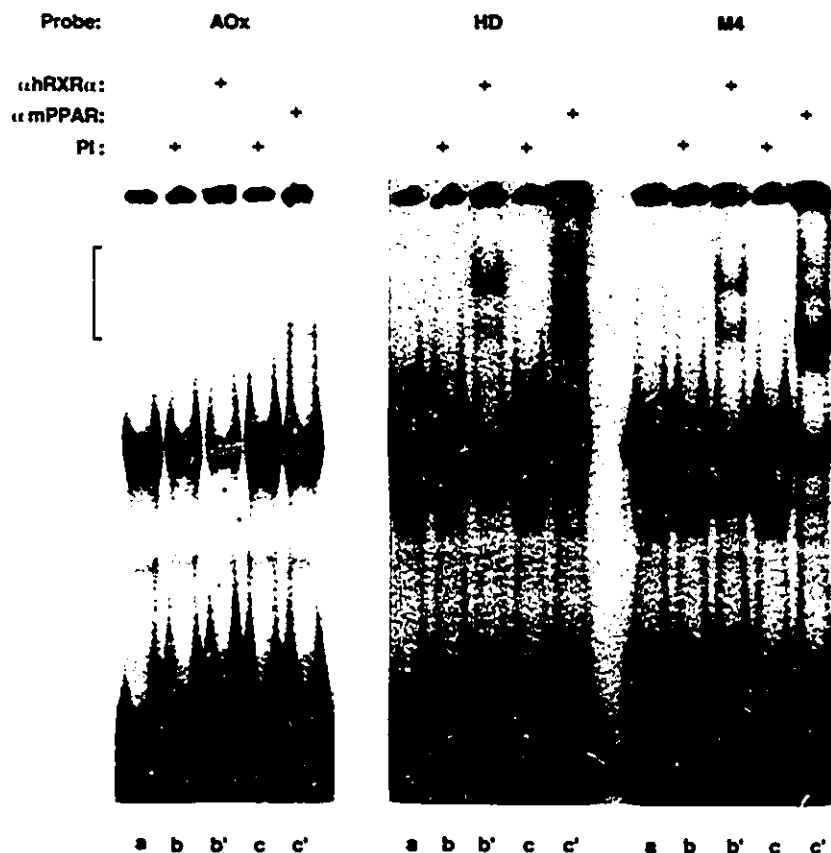


Fig. 3.14 RXR α and PPAR immunoreactivity of H4IEC3 protein complexes on the HD PPRE. Oligonucleotides of the wild type HD PPRE, the mutant M4, and the wild type AOx PPRE were radiolabeled. Nuclear extracts from H4IEC3 cells were preincubated either with BSA (lanes a), or with IgG (+) prepared from the preimmune (PI, lanes b and c), anti-hRXR α (α hRXR α , lanes b'), or anti-mPPAR serum (α mPPAR, lanes c'). The bracket indicates complexes retarded by the corresponding antibodies.

experiments with a mutant HD PPRE probe in which the last repeat was mutated so as to retain only the DR2 structure. This probe failed to interact with PPAR/RXR α translated *in vitro* or contained in the endogenous proteins of H4IIEC3 cell lysates.

Thus, the presence of an additional protein binding motif in the HD PPRE suggests that the PPAR/RXR α complex could function cooperatively with other nuclear receptors (Zhang *et al.*, 1993). DR2 repeats have been described in the CRABPI gene (cellular retinoic acid binding protein) and the CRABPII gene, which can be recognized by the heterodimer of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (Durand *et al.*, 1992). A DR1-containing element in the promoter region of the CRABPI gene is probably the target of RXR α (Durand *et al.*, 1992). In addition, it has been pointed out (Zhang *et al.*, 1993) that the arrangement of two TGACCT direct repeats separated by one single nucleotide, as in both the AOx and HD elements, is similar to the HNF-4 and COUP-TF1 binding sites in several genes (Sladek *et al.*, 1990; Mangelsdorf *et al.*, 1991; Kliewer *et al.*, 1992a). Since cooperative interactions among multiple nuclear factors could potentially generate diversity in signal transduction, it will be interesting to examine the nature of the receptor crosstalk on the HD PPRE and on the PPREs of other peroxisome proliferator-responsive genes.

3.3.4 *PPAR/RXR α Binding is Required, but not Sufficient, for In Vivo Transactivation of the HD PPRE*

To understand the role of each TGACCT half site in the HD element in PPAR/RXR α recognition and subsequent transactivation, mutations were introduced into each individual TGACCT half site of the HD PPRE. The oligodeoxyribonucleotides, M4, M3, and M5, carry mutations in TGACCT repeat 1, 2, and 3, respectively. M6 has a deletion of a spacer nucleotide between the first and second repeat. These mutant oligonucleotides were fused with the 5' end of the CPS proximal promoter in pCPS luc . Transient transfection assays in H4IIEC3 cells were done to assess the response of the mutated PPREs to ciprofibrate (Fig. 3.15). The activities of the wild-type HD PPRE oligonucleotide in pHD(X1) luc and pHD(X3) luc were induced approximately 3- and 18-fold, respectively, in the presence of ciprofibrate, similar to what has been shown previously. Constructs containing either one copy or three tandem copies of a mutant HD-PPRE oligonucleotide were unresponsive to ciprofibrate. These results were analysed together with data from DNA binding assays with radiolabeled mutant oligonucleotides. The mutants of the second repeat (M3) and the third repeat (M5) almost completely lost the ability to interact with liver nuclear factors (see Fig. 3.11 for M3; Fig. 3.24 for M5), indicating that transactivation requires protein binding to the HD PPRE.

A dissociation between protein binding and *in vivo* transactivation was observed with two other mutations. First,

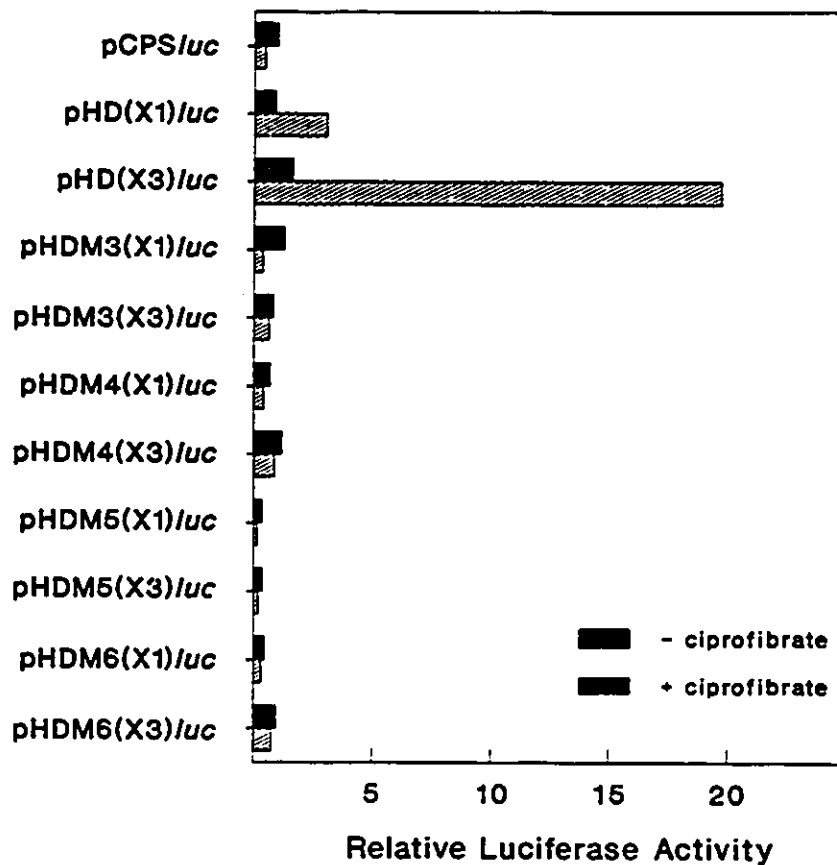


Fig. 3.15 HD PPRE mutants are unable to respond to ciprofibrate *in vivo*. H4IIEC3 cells were transfected with either the control vector *pCPSluc*, or plasmids with one copy (X1) or three copies (X3) of the oligonucleotides of the wild type HD PPRE (-2956 to -2919) or its mutants (M3, M4, M5, and M6, see Materials and Methods for sequences). Ciprofibrate (shaded bar) or dimethyl sulfoxide (dark bar) were added to the media. The values are averaged from two independent transfections, each done in duplicate, and are normalized to the activity of *pCPSluc* without ciprofibrate.

deletion of a spacing residue between the first and second TGACCT half site (M6) led to a complete loss of response to ciprofibrate [pHDM6(X1)luc and pHDM6(X3)luc in Fig. 3.15], while the oligonucleotide was still able to interact with the liver factors that bound to the wild type PPRE (Fig 3.16, M6, lane with added H4IIEC3 protein). However, the HD PPRE binding proteins were obviously subjected to competition by other DNA binding proteins that were not specific for a true HD PPRE. This was shown by the extra complexes formed on the M6 probe with rat hepatoma cell lysate, as well as with Rat2 and HeLa cell lysates, as compared to the corresponding lanes of the wild type probe. These results are in concordance with the current knowledge that the number of spacer nucleotides between TGACCT half sites is a critical determinant of specificity of a cis element (Umesono et al., 1991).

Second, mutation of the first repeat (M4) preserved largely its specificity to interact with the rPPAR/RXR α heterodimer. In fact, the liver protein complexes formed on the M4 probe were almost identical to those seen with the wild type probe (Fig. 3.14, compare the three lanes labeled with 'a'). This mutant, however, failed to respond to the ciprofibrate treatment *in vivo* (Fig. 3.15 pHDM4(X1)luc and pHDM4(X3)luc). Thus, although necessary, PPAR/RXR α binding is not sufficient for peroxisome proliferator-mediated induction via the HD PPRE. The integrity of all three direct TGACCT repeats and the correct spacing between the repeats in the HD PPRE are required for responsiveness to peroxisome proliferators.

The requirement for the first TGACCT motif implies that other transcription factors may be required for a coordinated interplay with the PPAR/RXR α dimer. Further, it differentiates the HD PPRE from the AOx PPRE. The two PPRES may either require different accessory transcription factors besides PPAR and RXR α or bind the same protein complexes with different affinities. Actually, both the AOx PPRE and the HD PPRE have been shown to require additional cellular components besides PPAR and RXR α to respond to peroxisome proliferators. Gearing et al. (1993) reported that additional factor(s) that were distinct from rRXR α were detected in COS-1 cells and enabled rPPAR to activate AOx PPRE. Studies of the HD PPRE by Marcus et al. (1993) have led to a similar conclusion. Marcus et al. also showed that the HD and AOx PPRES respond to the three *Xenopus* PPARs differently. For example, xPPAR γ may or may not result in a ciprofibrate induction, depending on which PPRE was co-introduced into the cells. Also, xPPAR γ -mediated responsiveness could be antagonized by xPPAR α or rPPAR. The DR1 component in the HD PPRE is, therefore, not an equivalent of the AOx element. The presence of an additional TGACCT motif may render the HD element able to receive different accessory nuclear factors and thus subject HD PPRE to a slightly different mechanism of gene regulation.

3.4 Role of Auxiliary Factors in Peroxisome Proliferator Signaling

3.4.1 A Distinct HeLa Factor Binds to the HD PPRE

Substantial differences between the AOx and HD PPREs in protein binding specificity were observed in mobility shift assays using HeLa nuclear lysate prepared by a detergent free protocol (Fig. 3.16). HeLa factors formed a major complex with the HD PPRE. A similar band could be seen with the AOx PPRE only after prolonged exposure. The mutant HD PPRE oligonucleotide M4 carrying a mutation in the first TGACCT motif so that it mimicks the AOx element was not recognized by HeLa factors. The DNase I footprint analysis of the 196-bp probe of the minimal HD PPRE with HeLa cell extract showed a footprint pattern almost identical to that formed with H4IIEC3 nuclear extract (Fig. 3.17). However, antiserum against hRXR α or mPPAR has never been able to detect the presence of either receptor in the complex between the HD PPRE and HeLa factors (data not shown). It appears, therefore, that a complex distinct from the RXR α /PPAR dimer is formed on the HD PPRE with HeLa extract and that the HeLa factor(s) requires the first TGACCT motif to bind to the element.

In order to address the possible role of this HeLa factor in transactivation, co-transfections of both rPPAR and hRXR α with pHD(X3)luc into BSC40, Rat2, and HeLa cells were conducted. Without co-expression of exogenous rPPAR and hRXR α , none of these cell

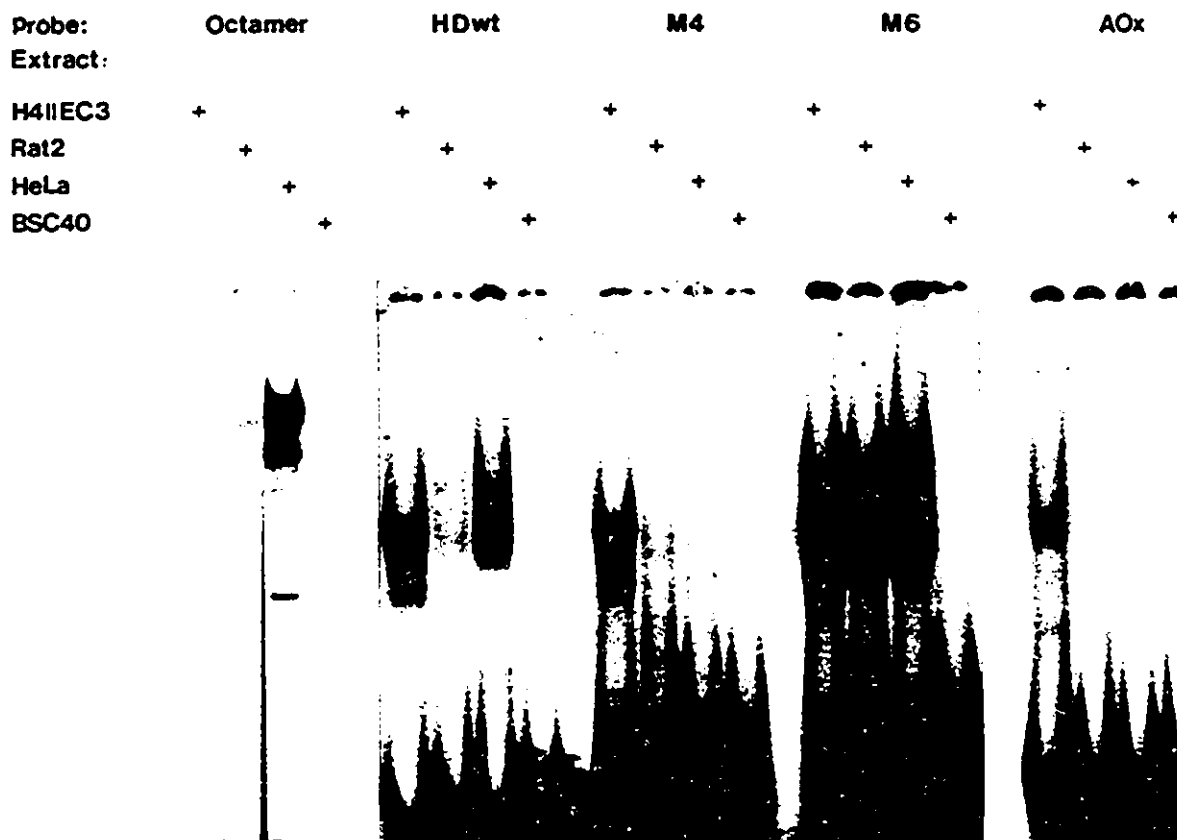


Fig. 3.16 The HD PPRE binding proteins in HeLa cells do not interact with the AOx PPRE or the HD mutant M4 in mobility shift assays. HDwt is the oligonucleotide of the wild type HD PPRE; M4, mutant in its first TGACCT motif; M6, deletion of the first spacing nucleotide (see Materials and Methods for sequences). AOx is the oligonucleotide of the AOx PPRE. Octamer contains a binding site for the nuclear protein, Oct-1 (see Fig. 3.4 for sequence). These oligonucleotides were radiolabeled and incubated with nuclear extracts from H4IIEC3, Rat2, HeLa, and BSC40 cells (+). The AOx PPRE binding reactions were electrophoresed in a second gel. The Oct-1 binding gel was exposed to X-ray film for 1 hr, while the others were exposed for 13 hr.

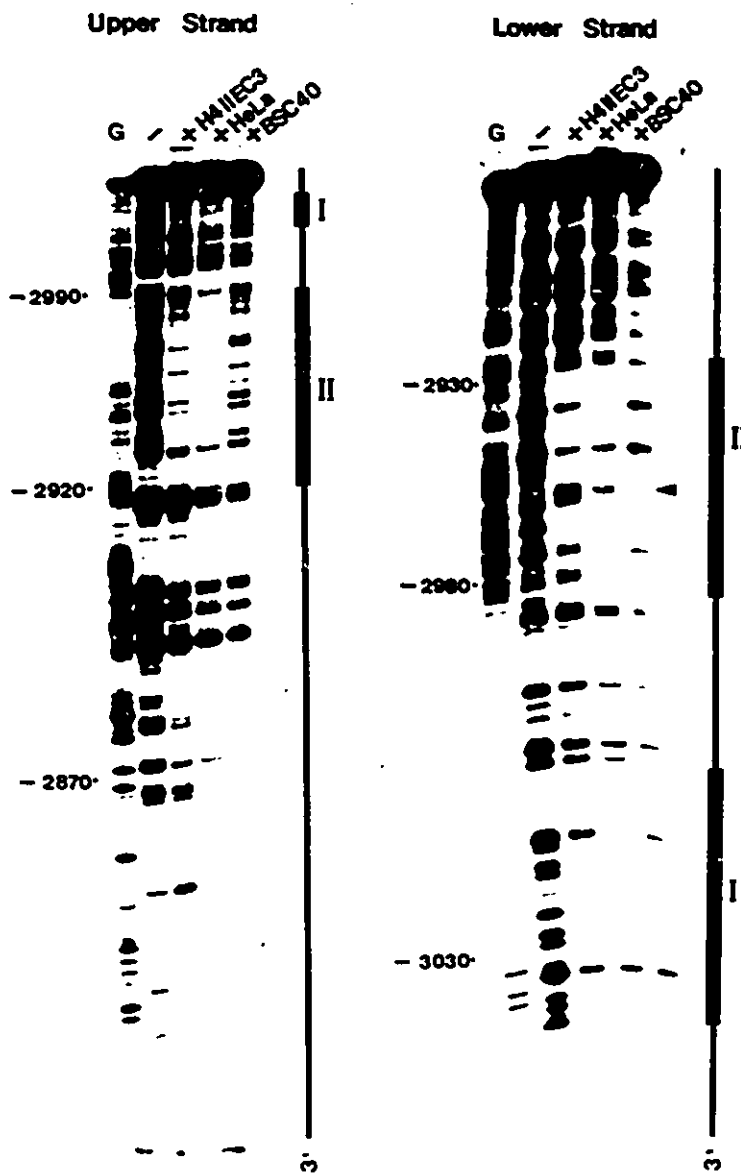


Fig. 3.17 DNase I footprint pattern of the minimal HD-PPRE binding proteins in HeLa cells. The 196-bp fragment of the minimal HD PPRE (-3040 to -2845) was radiolabeled at the 3'-end of either the *Upper Strand* or the *Lower Strand* and was incubated with BSA (-) or nuclear extracts from *H4IIEC3*, *HeLa*, or *BSC40* cells. The numbers correspond to the nucleotide positions determined by a G reaction of chemical DNA sequencing (G). The dark boxes I and II are regions protected from DNase I digestion by nuclear proteins.

lines is responsive to peroxisome proliferators (Fig. 3.18). In BSC40 and Rat2 cells, co-expression of both hRXR α and rPPAR was required for a significant ciprofibrate induction to occur. This is probably due to the absence of endogenous PPAR and RXR α in these cell lines since, in a preliminary test, the BSC40 nuclear lysate was not able to shift the radiolabeled HD PPRE oligonucleotide unless the lysate was made from cells pre-transfected with both hRXR α and rPPAR cDNA plasmids (data not shown).

In contrast, when pHD(X3)luc was co-transfected into HeLa cells with the rPPAR expression plasmid, a 2.3-fold induction upon ciprofibrate treatment was observed (Fig. 3.18). This is similar to the reported response of the AOx PPRE to the three xPPARs in HeLa cells (Dreyer et al., 1992) or the response of the HD PPRE and the AOx PPRE to rPPAR in COS-1 cells (Marcus et al., 1993; Gearing et al., 1993), supposedly due to the presence of an endogenous RXR α in both cell lines. However, unlike these reported results showing that addition of exogenous RXR α showed little effect, co-transfection of pHD(X3)luc with both rPPAR and hRXR α expression plasmids into HeLa cells failed to stimulate a response to ciprofibrate. Taken together, these data suggest that the HD-PPRE binding factor in HeLa cells may interfere with the up-regulation mediated cooperatively by exogenous hRXR α and rPPAR.

3.4.2 *The HeLa Factor is a COUP-TF1 Related Protein*

Sequence comparison of several hormone receptor binding sites has

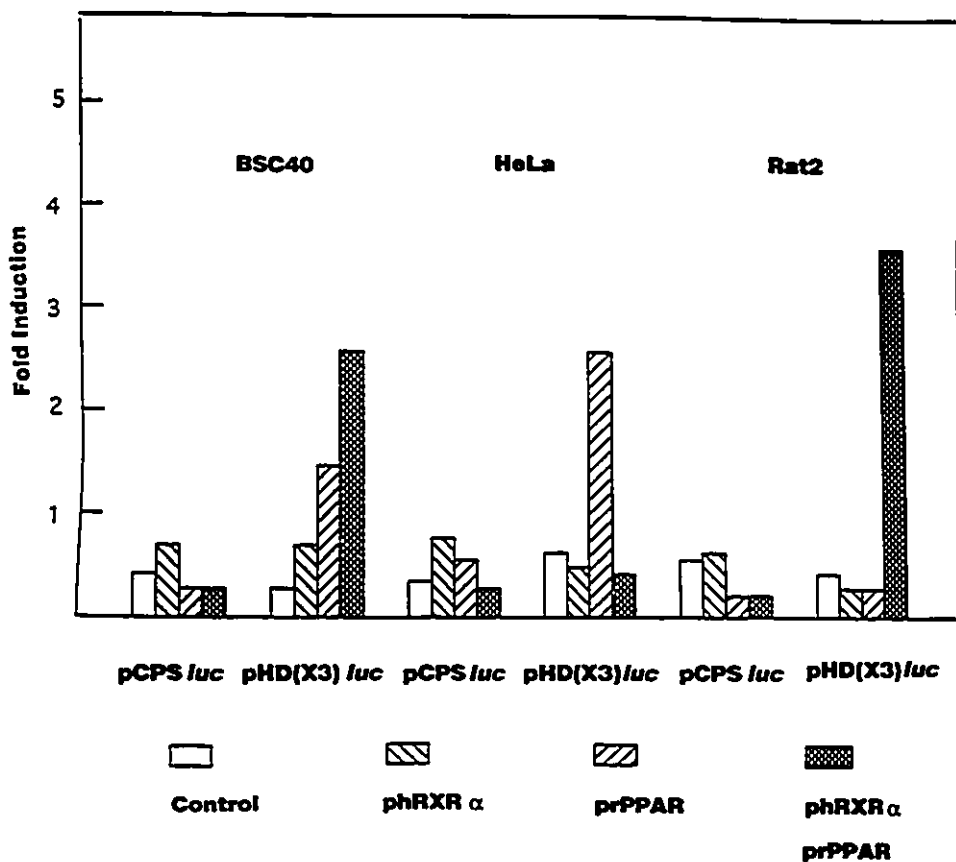


Fig. 3.18 Tissue-specific requirements of exogenous PPAR or RXR α for ciprofibrate-responsiveness. *pHD(X3)luc* was co-transfected into BSC40, HeLa, and Rat2 cells with either the empty vectors (control, see Materials and Methods for the respective vectors), or expression plasmids for rPPAR and hRXR α (*phRXR α* , *prPPAR*) in the presence or the absence of ciprofibrate. *pCPSluc* is a negative control vector for *pHD(X3)luc*. The integrated light units of luciferase activity are normalized to the value from transfections of *pCPSluc* without ciprofibrate. *Fold induction* is the ratio of the luciferase activity in the presence of ciprofibrate over that in its absence.

predicted that the HD PPRE as well as the AOX PPRE could be recognized by some auxiliary factors, such as hepatic nuclear factor (HNF-4) and chicken ovalbumin upstream promoter-transcription factor (COUP-TF) (Zhang et al., 1993). These are orphan receptors and members of the steroid receptor superfamily (Lucas and Granner, 1992; Sladek et al., 1990). Whereas both are involved in the regulation of several genes related to lipid homeostasis, COUP-TF proteins have been shown to be negative regulators of the retinoic acid-responsive pathways (Tran et al., 1992).

To explore whether members of the COUP-TF family are present in the complexes of HeLa and H4IIEC3 proteins with the HD PPRE, a mobility shift experiment was carried out in the presence of a polyclonal antibody against hCOUP-TF1, radiolabeled HD PPRE oligonucleotide, and nuclear extracts prepared from H4IIEC3 and HeLa cells (Fig. 3.19). This antibody recognizes both COUP-TF1 and a related receptor COUP-TF2 (Cooney et al., 1992), which is also called ARP-1 (Ladiaz et al., 1991). A major protein-DNA complex formed between the HD PPRE and HeLa cell nuclear proteins was almost quantitatively supershifted by anti-hCOUP-TF1 (Fig. 3.19.A, lane c). The fact that the HD PPRE binding species in HeLa cells is predominantly composed of COUP-TF-related proteins agrees with the negative feature of the HeLa factor in cell transfection studies.

Interestingly, addition of anti-hCOUP-TF1 also resulted in the

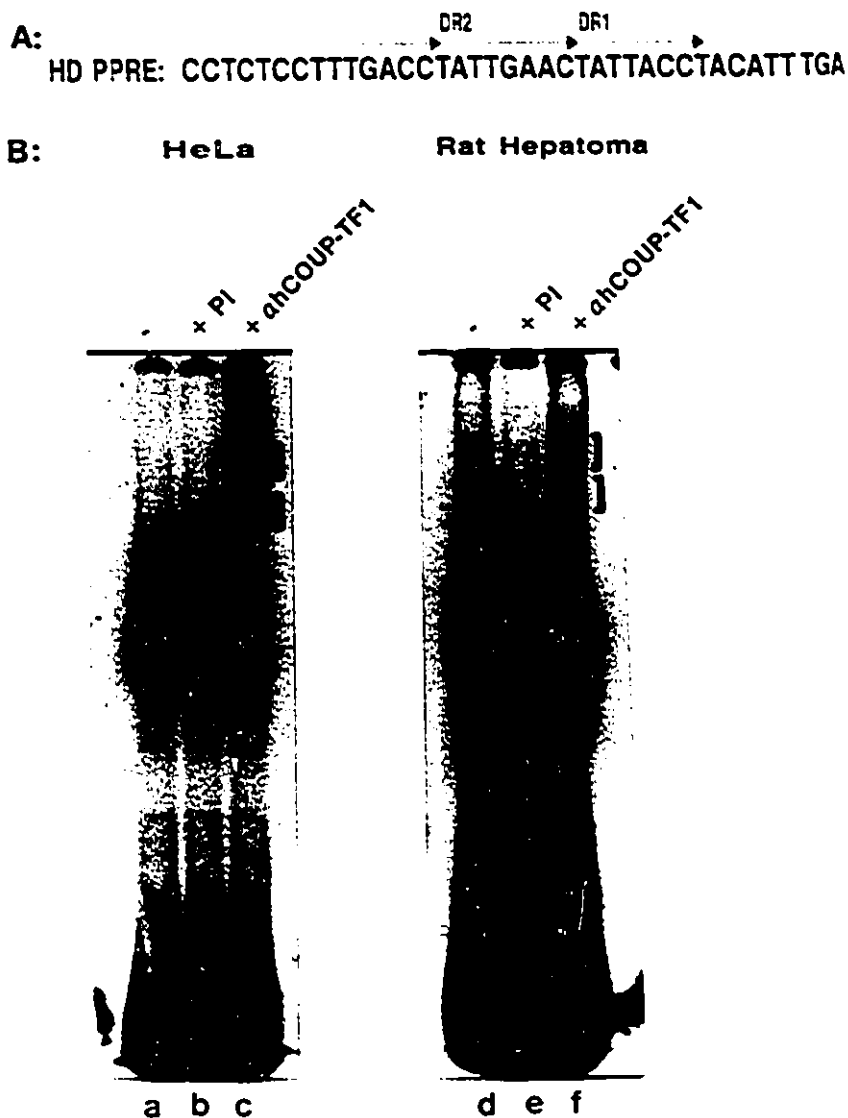


Fig. 3.19 COUP-TF1-related factor is present in the protein-DNA complexes of HeLa and H4IIEC3 lysates with the HD PPRE. (A) The core HD PPRE oligonucleotide sequence. The arrow designates the direct repeats of the TGACCT motifs. DR1 and DR2 mean direct repeats separated by 1 nucleotide and 2 nucleotides, respectively. (B) Mobility shift assay with the radiolabeled HD PPRE oligonucleotide. The nuclear extracts from HeLa (left panel) and Rat Hepatoma H4IIEC3 (right panel) cells were preincubated with 5 μ g of IgG prepared from preimmune (+PI, lanes b and e) or anti-hCOUP-TF1 serum (+ahCOUP-TF, lanes c and f), or with an equal amount of BSA (-). The brackets indicate the complexes retarded due to the presence of the anti-hCOUP-TF1.

formation of supershifted complexes with lysates from rat hepatoma cells (Fig. 3.19.B, lane f). It appears, therefore, that proper functioning of the RXR α /PPAR dimer depends on the proper context of these and other auxiliary factors in the cell.

3.4.3 *COUP-TF1 and HNF4 Differentially Interact with the HD and AOx PPREs In Vitro*

The presence of COUP-TF1 in the protein-PPRE complex raised our curiosity to examine the possible interplay among nuclear proteins. The IgG preparations of anti-hCOUP-TF and anti-rHNF4 serum were used in mobility shift assays with H4IIEC3 nuclear extract and radiolabeled oligonucleotides of HD and AOx PPRE, and the HD mutant oligonucleotide M4 with its first TGACCT motif mutated (Fig. 3.20). Both antibodies supershifted components in the complexes with the AOx PPRE (brackets). Surprisingly, no supershifted species were observed with the M4 probe although it retained a DR1 arrangement similar to the AOx PPRE. In addition, little HNF4 was detected in the protein complex with the wild type HD oligonucleotide. Therefore, the DR1 in the HD PPRE is not equivalent to the DR1 in the AOx PPRE. COUP-TF1 and HNF4 appear to demand an accurate primary nucleotide sequence in the target site.

Both rHNF4 and hCOUP-TF1 were capable of binding to the TGACCT direct repeats in HD PPRE independent of PPAR and RXR α in a mobility shift experiment carried out with *in vitro* translated receptors (Fig. 3.21). Consistent with the mobility shift data in



Fig. 3.20 COUP-TF1- and HNF4-related proteins are present in the protein-PPRE complexes. Oligonucleotides of the wild type HD PPRE (HD), the mutant of its first repeat (M4), and of the AOx PPRE (AOx) were radiolabeled and incubated with nuclear extracts from H4IIEC3 cells in the presence of BSA (lanes a), the preimmune (lanes b) and immune (lanes c) serum of anti-rHNF4 (α HNF4, lower panel), and the IgG extract from COUP-TF1 pre- (lanes b) and anti- (lanes c) serum (α hCOUP-TF1, upper panels). The arrows indicate supershifted complexes. The preimmune serum resulted in a nonspecific band. The anti-rHNF4 serum was not purified because only a limited amount of it was available.

the presence of the corresponding antibodies, the AOX PPRE was recognized by both proteins, but much less intensely by hCOUP-TF1, when compared with the HD PPRE. The HD PPRE wild type sequence showed high affinity to hCOUP-TF1 and little affinity to rHNF4. Interestingly, the first repeat mutant (M4) that retained a DR1 structure as well as the ability to bind PPAR/RXR α failed to bind either COUP-TF1 or HNF4. A conclusion can be drawn that, in contrast to the simple DR1 requirement for the cooperative binding of PPAR and RXR α , the accessory proteins COUP-TF1 and HNF4 rely more on the internal nucleotide sequence of the binding site.

To further define the nucleotide sequence requirements within the HD PPRE for the binding of COUP-TF1, versus the cooperative binding of PPAR and RXR α , mobility shift experiments were performed using mutant oligonucleotides with the three TGACCT-like direct repeats of the core HD PPRE individually mutated (Fig. 3.22). Mutation of the first repeat (M4) had no effect on the binding of *in vitro* translated rPPAR/RXR α (lane c) but completely abolished COUP-TF1 binding (lane d). A single nucleotide mutation in the second repeat (M3) significantly reduced binding of both COUP-TF1 and rPPAR/RXR α (lanes e and f, respectively). Mutation of the third repeat (M5) almost abolished rPPAR/hRXR α binding (lane g) but hardly affected COUP-TF1 binding (lane h). Therefore, COUP-TF1 and rPPAR/RXR α recognize distinct, yet overlapping, core elements within the HD PPRE. The first two repeats are important determinants for COUP-TF1 binding, and the second and third repeats

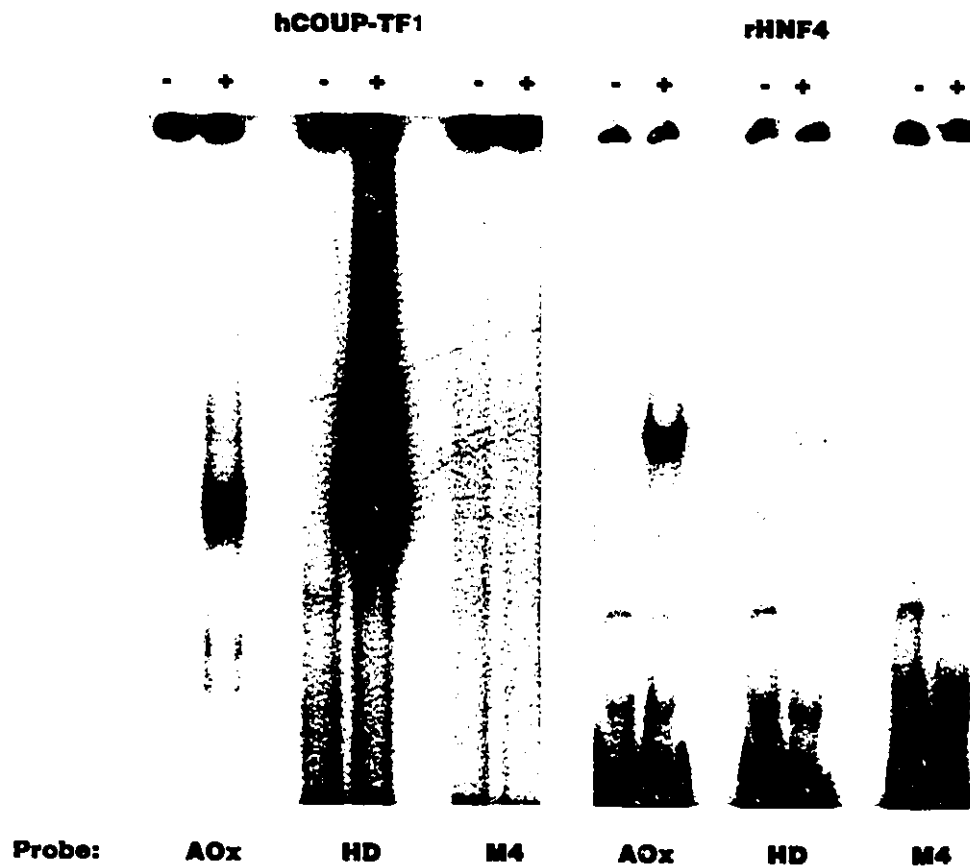


Fig. 3.21 *In vitro* translated COUP-TF1 and HNF4 bind to the HD and AOx PPREs. Oligonucleotides of the wild type HD PPRE (HD), the mutant of its first TGACCT motif (M4), and the AOx PPRE (AOx) were radiolabeled and incubated with *in vitro* translated hCOUP-TF1 or rHNF4 (+). Unprogrammed rabbit reticulocyte lysate was used as a negative control (-).

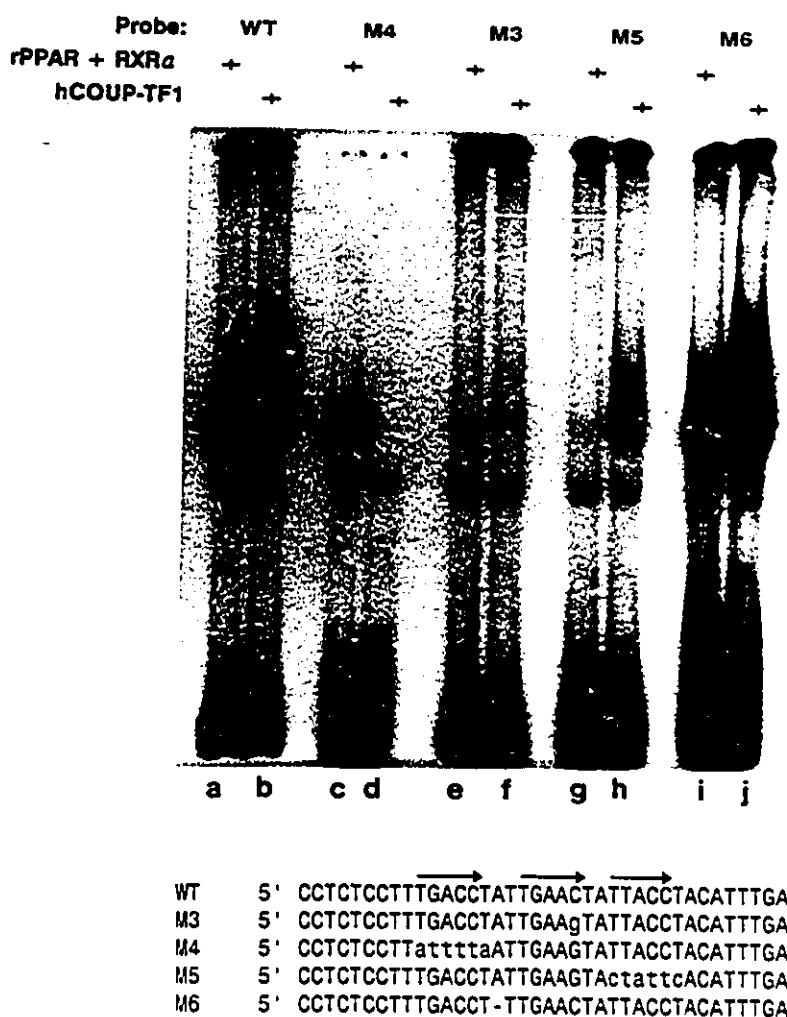


Fig. 3.22 The three TGACCT-like motifs in the HD PPRE display different affinities for PPAR/RXR α and COUP-TF1. The HD PPRE oligonucleotide probe corresponds to nucleotides -2956 to -2919 (WT). The probes M4, M3, and M5 carry mutations in the first, second, and third TGACCT motif of the HD PPRE, respectively; M6 has a deletion of the A residue between the first two repeats of the HD PPRE. The probes were incubated with *in vitro* translated products of either rPPAR plus RXR α , or hCOUP-TF1 alone. The nucleotide sequences of the probes are shown at the bottom. The arrows indicate the TGACCT motifs.

are necessary for the cooperative binding of rPPAR and RXR α . The nucleotide spacing between two repeats is not a strict requirement for COUP-TF1 binding since deletion of a spacer nucleotide which converted the DR2 in the HD element to a DR1 strongly bound COUP-TF1 (M6, lane j). Therefore, the primary sequence of the first two repeats, and not their relative spacing, is important for binding of COUP-TF1. This observation is consistent with the reported promiscuous recognition of COUP-TF proteins for several HREs with variant numbers of spacing nucleotides between the half-sites (Tran et al., 1992).

3.4.4 *COUP-TF1 Antagonizes Peroxisome Proliferator-Mediated Signaling*

COUP-TFs repress transcription induction by hormone receptors through the formation of inactive DNA-binding heterodimers (Cooney et al., 1993). To determine whether COUP-TF1 could downregulate PPAR/RXR α -mediated transactivation *in vivo*, the pHD(X3)*luc* reporter plasmid was co-transfected with expression plasmids for rPPAR, RXR α , and COUP-TF1 into BSC40 cells (Fig. 3.23). BSC40 cells were chosen, because they are unresponsive to peroxisome proliferators without co-transfection of PPAR and RXR α expression plasmids and do not contain factors that bind to the HD PPPE. A 10-fold induction in luciferase activity was observed in the presence of both rPPAR and RXR α , which was further increased by the addition of ciprofibrate. Addition of increasing amounts of COUP-TF1 expression plasmid alone had no effect on the basal activity of pHD(X3)*luc*.

However, co-expression of COUP-TF1 with the rPPAR and hRXR α expression plasmids exerted an inhibitory effect on ciprofibrate induction. This inhibitory effect was quantitatively dependent on the amount of COUP-TF1 expression plasmid, which was consistent with the inhibitory effect of COUP-TF proteins on other transactivators, such as HNF4 (Cooney et al., 1993). It is concluded, therefore, that COUP-TF1, antagonizes PPAR/RXR α -mediated peroxisome proliferator signaling *in vivo*.

Growing evidence has shown the importance of auxiliary factors in gene regulation. COUP-TF has been shown to be a negative regulator of genes involved in lipid metabolism, such as several apolipoprotein genes (Ladiaz et al., 1992; Snyder et al., 1992; Metzger et al., 1993). The physiological significance of COUP-TF proteins is that they are the only known negative regulators that act on many different HREs interacting with different transcription factors. In some cell culture co-transfection systems, COUP-TF acted as an antagonistic factor to HNF4 (Snyder et al., 1992). In other systems, COUP-TF behaved as a negative regulator by virtually repressing all retinoid X receptor-mediated hormonal induction, which was, in some case, via heterodimers with the universal positive co-regulator, the retinoid X receptor (Cooney et al., 1993). Data from our laboratories suggested that hCOUP-TF1-mediated antagonism occurred via competition of hCOUP-TF1 homodimer with PPAR/RXR α heterodimer for an occupancy of the DNA target site on the HD-PPRE (Miyata, et al., 1993). It is for this reason that some

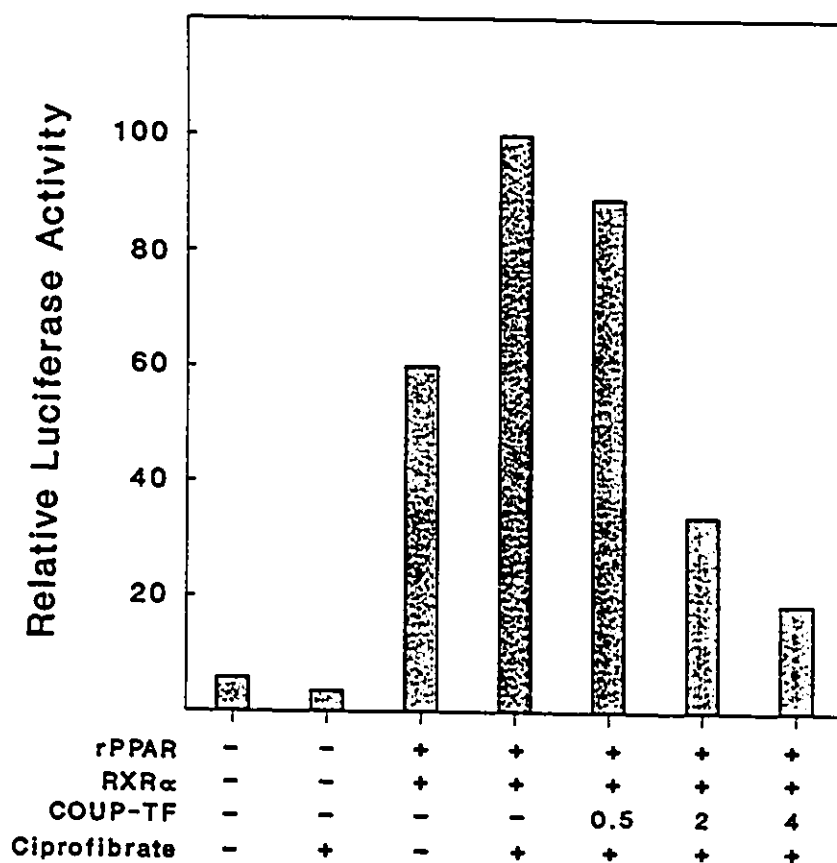


Fig. 3.23 COUP-TF1 antagonizes peroxisome proliferator-mediated signaling. pHD(X3)luc was transfected into BSC40 cells together with plasmids expressing rPPAR, hRXR α , and hCOUP-TF1. The numbers in the bottom legend are micrograms of plasmid DNA expressing hCOUP-TF added in each plate during transfection. The values of several transfections, each in duplicate, are averaged. Relative luciferase activity is the integrated light units normalized to the value of the control, a co-transfection of pCPSluc with the expression plasmids of these nuclear receptors.

scientists have postulated that COUP-TF may play a master role in these pathways (Cooney et al., 1993). Data presented in this thesis suggest that transcription of the peroxisomal HD gene depends upon the intracellular balance of these positive and negative regulatory factors. This sophisticated regulation provides a mechanism for the peroxisomal genes to respond to a variety of physiological and environmental stimuli.

3.5 Perspectives

3.5.1 *PPRE-Binding Factors Exist in Both the Cytoplasm and the Nucleus*

Mobility shift experiments using HeLa cell extracts prepared with or without the detergent, NP40, led to the results different from those reported in previous sections. It raised the question whether NP40 was having an effect in these mobility shift experiments. H4IIEC3 cells were fractionated into cytosolic and nuclear extracts by Dignam's method (Dignam et al., 1983) without drugs or detergent. The cytosolic and nuclear fractions were assayed for PPRE binding in mobility shift experiments using radiolabeled oligonucleotides of the wild type HD PPRE or the mutant HD PPRE oligonucleotides M3, M4, M5, or M6.

Surprisingly, some cytosolic proteins were able to recognize this nuclear DNA enhancer sequence (Fig. 3.24). The wild type HD PPRE oligonucleotide could form at least two major complexes with

cytosolic proteins and two with nuclear proteins (Fig. 3.24, right panel). The octamer probe with a binding site for the ubiquitous nuclear protein, Oct-1, was used in parallel to test the quality of the compartment separation (Fig. 3.24, left panel). Only the nuclear extract shifted the Oct-1 probe, indicating that contamination of the cytosolic fraction by nuclear protein is negligible. An additional complex resulted by mixing the extracts from the two compartments, which, in fact, indicated that the nuclear fraction was not greatly contaminated by cytosolic proteins. In the case of poor separation, this additional complex would appear also in the putative nuclear fraction (data not shown).

Comparison of the corresponding lanes of the wild type HD PPRE oligonucleotide with those of the repeat-1 mutant (M4) led to the identification of one cytosolic and one nuclear protein complex that were absent with the disruption of the DR2 structure in the HD PPRE (Fig. 3.24, M3 and M4). These results indicate the presence of other DR2-mediated HD-PPRE binding proteins in H4IIEC3 cells. They are not the same as COUP-TF1, since retaining the DR2 (M5) did not retain efficient binding of these protein complexes, while M5 was able to interact with COUP-TF1 as efficiently as the wild type HD oligonucleotide. In view of the highly specific recognition between the HD PPRE and rat liver factors, it would be interesting to characterize these novel complexes in further detail and to investigate the possibility that they may mediate the response of

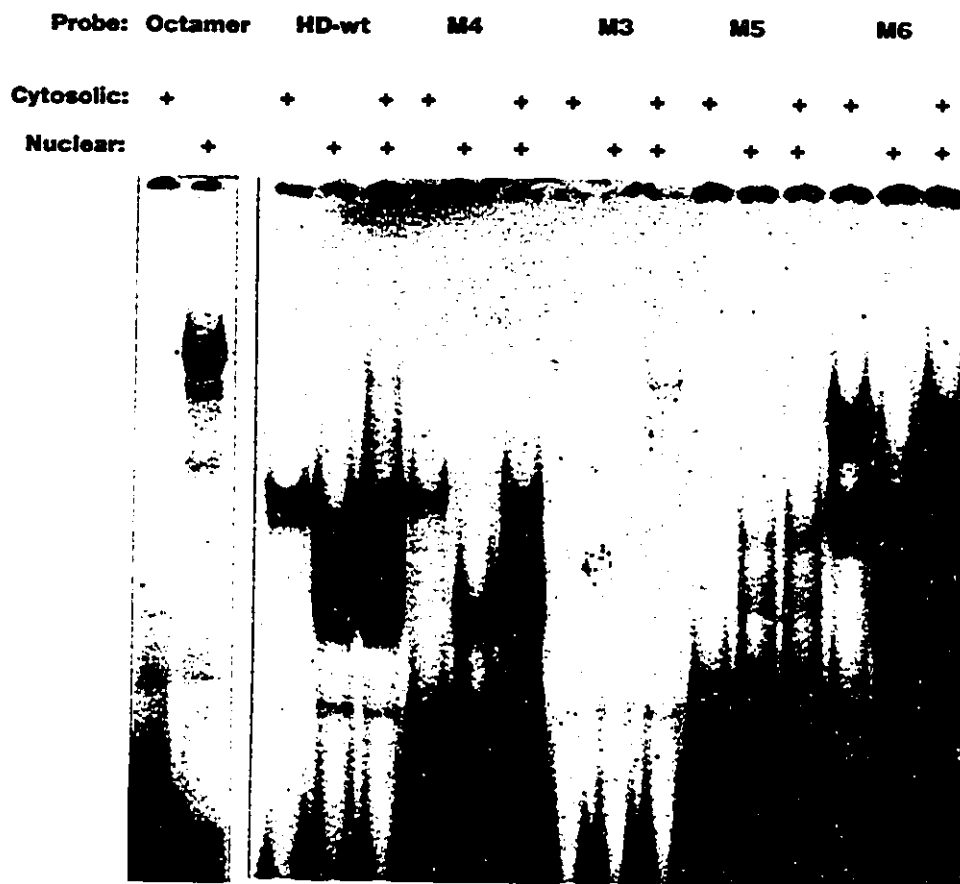


Fig. 3.24 . HD-PPRE binding proteins are present in both the cytoplasm and the nucleus. Mobility shift assays were done with the radiolabeled oligonucleotides of the wild type HD PPRE (HD-wt), mutants of its first (M4), second (M3), or third (M5) TGACCT motif, or a mutant with a deletion of the A residue between its first and second repeats (M6). H4IIEC3 cell lysates were separated into cytosolic and nuclear fractions, and the proteins were extracted separately by Dignam's method (1983). Every three lanes in the right panel represent experiments with one oligonucleotide probe incubated with 5 μ g of either cytosolic (every first lane) or nuclear (every second lane) extract, or a mixture of 5 μ g of each extract (every third lane). The octamer probe (see Fig. 3.4 for sequence) contains a binding site for the nuclear protein Oct-1 and is here to show that the cytosolic fraction is free of contamination by the nuclear fraction.

the HD PPRE to ciprofibrate and other peroxisome proliferators.

3.5.2 Cytoplasmic Isoforms of the Nuclear Transcription Factors

Since binding to DNA has biological relevance only in nucleus, a cytoplasmic protein must be able to translocate into nucleus in order to perform any function in gene regulation. Similar hypotheses have in the past led to the discovery of cytoplasmic isoforms of several transcription factors (Lenardo and Baltimore, 1989; Thanos and Maniatis, 1992). For example, in the absence of hormone, glucocorticoid receptor (GR) is a cytoplasmic protein in the form of an apo-receptor complexed with a heat shock protein (HSP90) (Scherrer et al., 1993). Upon binding of the steroid hormone, GR is released from the complex and migrates into the nucleus to bind to glucocorticoid response elements, thereby, acting as a transcription factor (Picard and Yamamoto, 1987).

This knowledge encouraged a further test of the cytosolic HD-PPRE binding proteins with polyclonal antibodies against hCOUP-TF1, rHNF4, hRXR α , and mPPAR (Figs. 3.25-3.27). Antibodies against hRXR α and mPPAR supershifted cytoplasmic complexes on the wild type HD PPRE and AOx PPRE (Fig. 3.27). A supershifted cytosolic species of rHNF4 was observed with the AOx PPRE (Fig. 3.26). Considering that there was little chance for the cytosolic extract to be contaminated by nuclear protein, the data suggest that RXR α , PPAR, and HNF4 have cytosolic isoforms. Supportive of this, these cytosolic forms seem to interact with the two PPREs in a fashion

similar to *in vitro* translated proteins. The anti-hRXR α and anti-mPPAR preparations clearly supershifted the same complexes formed between the wild type HD PPRE or its mutant M4 and the cytosolic fraction, indicating that a PPAR/RXR α dimer may be the major component of these complexes. The rENF4-related protein preferred the AOx PPRE but showed much less affinity to the wild type HD PPRE or its mutant M4 (Fig. 3.26).

The anti-hCOUPTF1 could recognize only the nuclear complex (Fig. 3.25, arrowhead). In contrast, anti-RXR α did not clearly supershift or reduce the nuclear complexes with any of the three probes (Fig. 3.27, bottom panels, center lane under each probe). The same anti-RXR α preparation had successfully and repeatedly shifted PPAR/RXR α in many different experiments. It is unlikely, therefore, that no supershift from nuclear extract with anti-RXR α was due to the IgG preparation. For similar reasons, it is less likely that RXR α was buried so that it is totally inaccessible to the antibody. It appears reasonable, therefore, to question the subcellular localization of this and other factors.

3.5.3 *Is There a Nuclear Translocation?*

The intracellular localization of these transcription factors has not been thoroughly investigated. Dreyer et al. (1993) showed a predominant nuclear localization of xPPAR α and β by immunocytochemistry. On the other hand, Gebel et al. (1992) reported that rPPAR was detected by Western blot analysis in rat

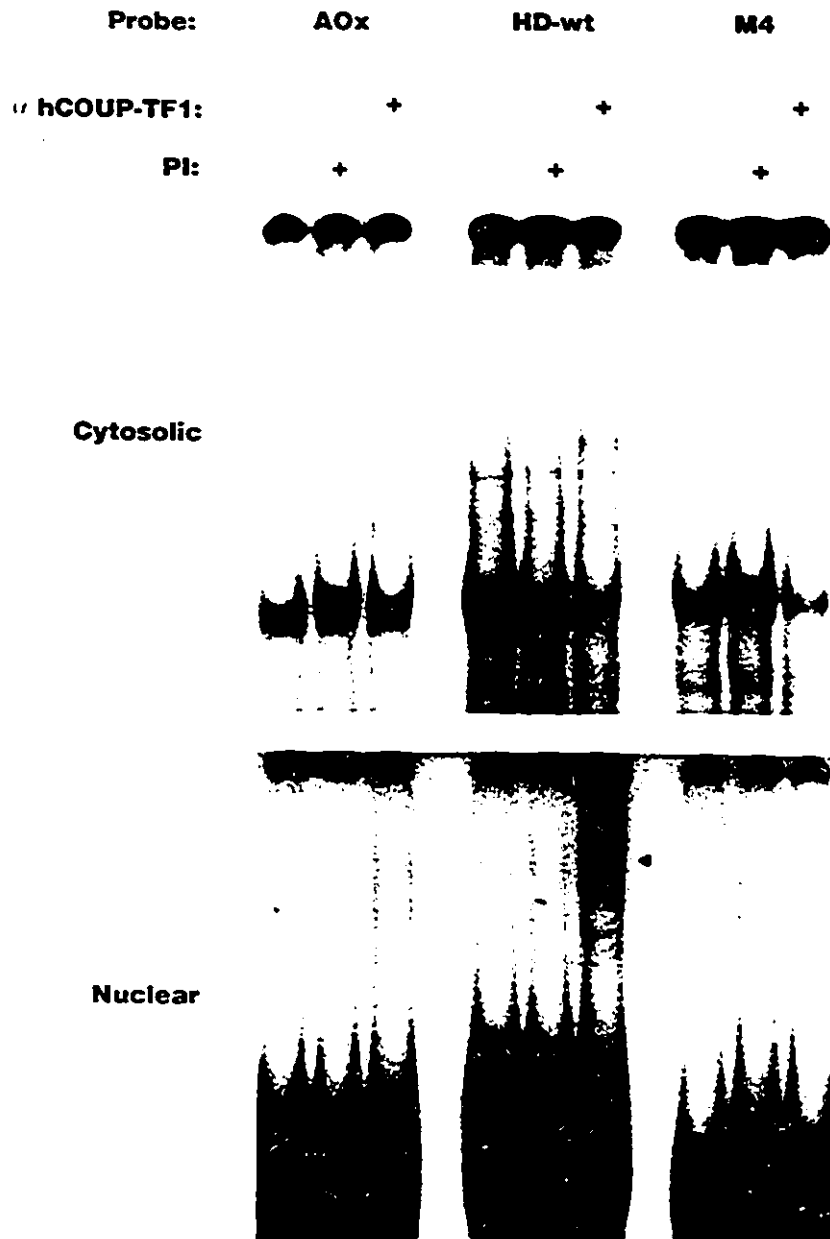


Fig. 3.25 COUP-TF1-related protein is present in complexes of nuclear proteins with the HD PPRE. Radiolabeled AOx PPRE, HD PPRE (HD-wt), and its mutant M4 (with its first TGACCT repeat mutated) were tested in a mobility shift assay. Cytosolic and Nuclear proteins were preincubated with 5 μ g of BSA (every first lane), IgG from preimmune (PI) or anti-hCOUP-TF1 serum (α hCOUP-TF1). The arrowhead points to the protein-DNA complexes supershifted by the anti-hCOUP-TF1.

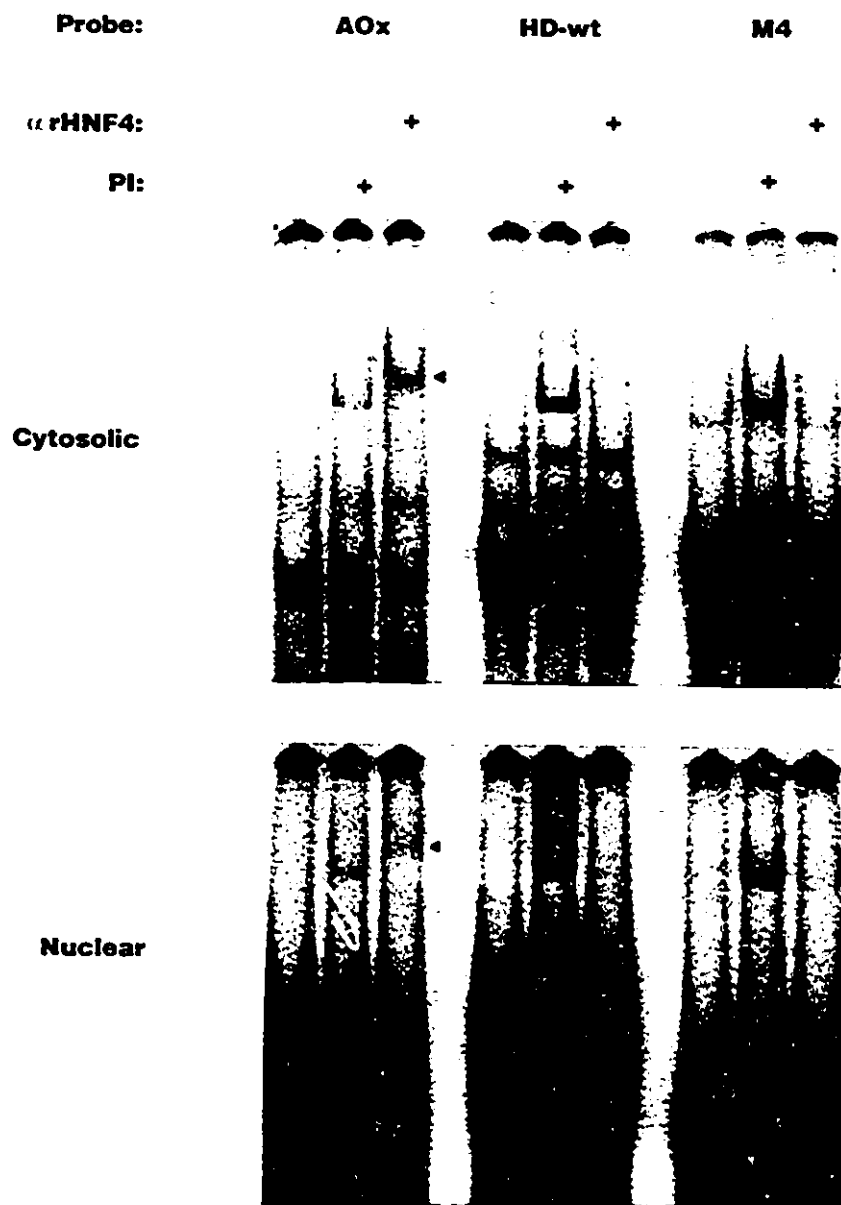


Fig. 3.26 HNF4-related protein is present in complexes of H4IEC3 proteins with AOx PPRE. The AOx PPRE (AOx), the HD PPRE (HD-wt) and a HD PPRE mutant with its first repeat mutated (M4) were tested in mobility shift assays. Preimmune (PI) or anti-rHNF4 (α rHNF4) serum were preincubated with the Cytosolic or Nuclear proteins of H4IEC3 cells. The arrowheads point to supershifted species. PI causes a nonspecific shift.

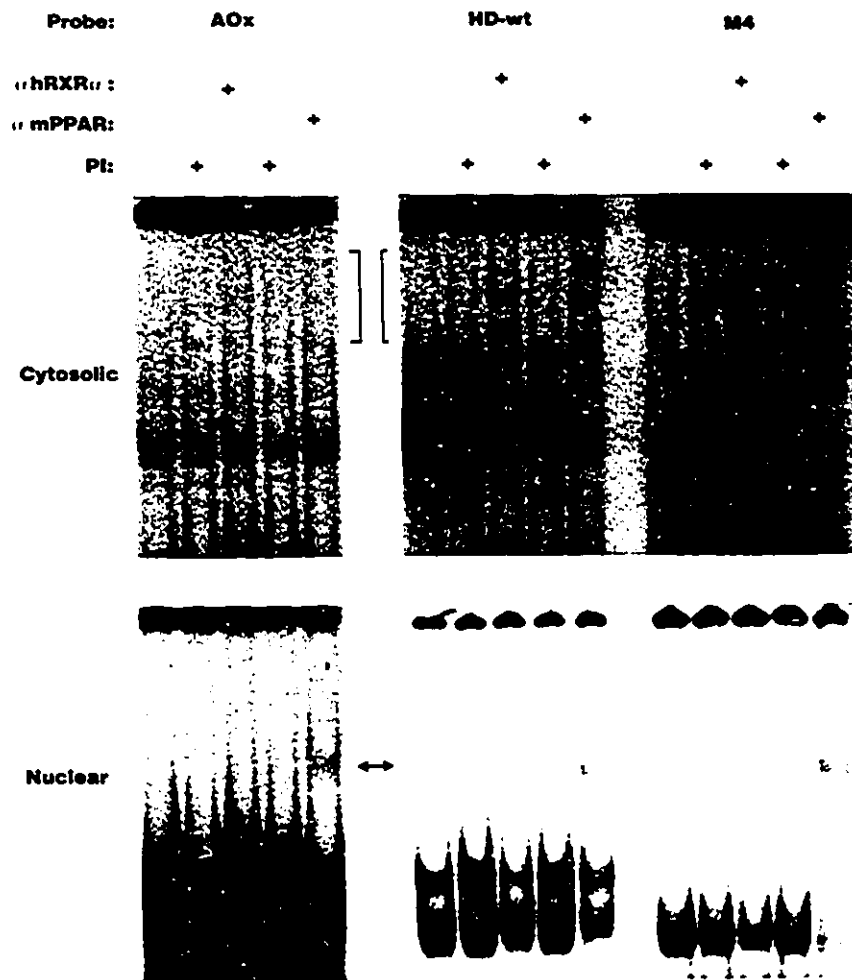


Fig. 3.27 PPAR-related protein is detected in complexes of nuclear and cytoplasmic proteins with a PPRE. Mobility shift assays were performed with radiolabeled AOx PPRE (AOx), HD PPRE (HD-wt), and a HD PPRE mutant with its first TGACCT repeat mutated (M4). Cytosolic or nuclear proteins of H4IIEC3 cells were preincubated with 5 μ g IgG purified from anti-hRXR α (α hRXR α), anti-mPPAR (α mPPAR) serum, or the respective preimmune sera (PI). Every first lane for each probe is a BSA control. The arrows and the brackets indicate the protein-DNA complexes reactive with the corresponding antibody preparation.

liver cytosol and nucleus after the animals were treated with a peroxisome proliferator.

Intracellular localization of RXR α has not been reported. Since I had liver cell nuclear extracts isolated from clofibrate-treated and untreated rats, a Western blot analysis was performed with anti-RXR α IgG preparation (Fig. 3.28). A protein species with a molecular size close to the predicted 51 kDa of rRXR α (Mangledorf et al., 1992; Gearing et al., 1993) was present in clofibrate-treated nuclear lysate but not in the untreated control lysate (arrow). Unfortunately, the cytoplasmic protein preparations were not well stored, having been prepared several years ago and the data were inconclusive as to whether or not the increased RXR α signal resulted from transcriptional regulation of an RXR α gene or from nuclear translocation of an RXR α -related protein from cytoplasm to the nucleus. This result is, however, not contradictory with those from mobility shift data in this thesis in that the HD PPPE-protein complex formed with nuclear extract prepared without addition of drug or detergent was not supershifted by the anti-hRXR α preparation. Considering the extensive role of this factor in hepatic metabolism, one would expect a constitutive presence of RXR α . In addition, RXR α -related protein was indeed detected in the untreated H4IIEC3 cell cytosol by mobility shift assay. These preliminary data point to an important area for future studies which would provide biochemical detail of the protein-DNA recognition observed in this thesis under various conditions.

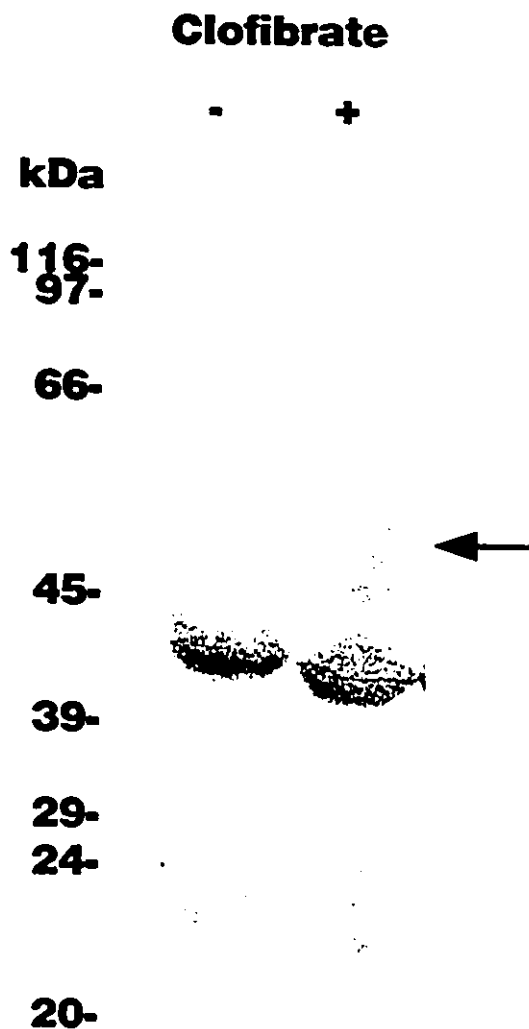


Fig. 3.28 Detection of anti-RXR α reactive protein in clofibrate-treated rat liver nuclei. Rats were treated with 0.5% clofibrate in diet (+) or not treated (-) for 15 days. Nuclear proteins of the hepatocytes were extracted as in Materials and Methods, separated by 10% SDS-PAGE, and transferred to nitrocellulose. The filter was blotted with IgG purified from anti-hRXR α serum and developed using the ECL system (Amersham Canada Ltd.). The sizes of molecular weight standards are given on the side (in kDa). The arrow points to an induced protein species with a molecular size close to the predicted 51 kDa of rRXR α (Mangledorf et al., 1992).

SUMMARY

Extensive evidence has shown that chemically induced peroxisome proliferation results from the up-regulation of transcription of peroxisomal genes, especially those encoding β -oxidation enzymes. Studies on the promoter of the gene encoding the first enzyme of the peroxisomal β -oxidation pathway has led to the isolation of an enhancer-like sequence. This AOx PPRE consists of two direct repeats of TGACCT-like motifs, a consensus sequence of the target sites for many members of the steroid receptor superfamily. Two members of this family: an orphan receptor, PPAR, and the 9-cis retinoic acid receptor, RXR α , from different species are able to bind to the AOx PPRE cooperatively and transactivate the gene in response to peroxisome proliferators.

Research presented in the thesis aimed at determining the mechanisms of transcriptional regulation of the gene encoding the second peroxisomal β -oxidation enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD). An HD PPRE was isolated from the promoter region of the HD gene. It consists of three direct repeats of the TGACCT-like motif separated by two nucleotides and one nucleotide, respectively. This HD PPRE confers responsiveness to peroxisome proliferators independently, although other enhancer-like elements flanking this core element seem to contribute to a maximal response. In a ciprofibrate-responsive rat hepatoma cell

line, the function of this PPRE is independent of its position and orientation relative to the transcription start site and is independent of the context of the proximal promoter. *In vitro*, it binds to liver-specific proteins. This DNA-protein recognition is shown to be highly nucleotide sequence-specific and is a prerequisite for transcriptional activation.

Multiple factors are involved in mediating the ciprofibrate-responsiveness of the HD PPRE. As schematically summarized by Dr. J. Capone (Fig. 3.29), the DR1 structure in the HD PPRE is the target sequence for the cooperative binding of PPAR and RXR α . Both rat PPAR and RXR α are present in the complex formed with the HD PPRE and rat liver nuclear proteins. Their cooperative binding to the HD PPRE is required for ciprofibrate-mediated transcriptional regulation *in vivo*. Accessory factors, however, are also involved. A HeLa factor could bind to the HD PPRE in a fashion similar to that of liver factors. Yet, the HeLa factor is not capable of mediating any response to ciprofibrate by itself and it is a hCOUP-TF1 immunoreactive protein. *In vitro* translated hCOUP-TF1 is able to bind to the DR2 structure in the HD PPRE in a fashion similar to that of HeLa factor. *In vivo*, like its HeLa relative, hCOUP-TF1 antagonizes the PPAR/RXR α -mediated up-regulation of a HD PPRE-driven gene.

Mutation of the first TGACCT motif in the HD PPRE preserved the binding of PPAR/RXR α dimer to the element but abolished its response

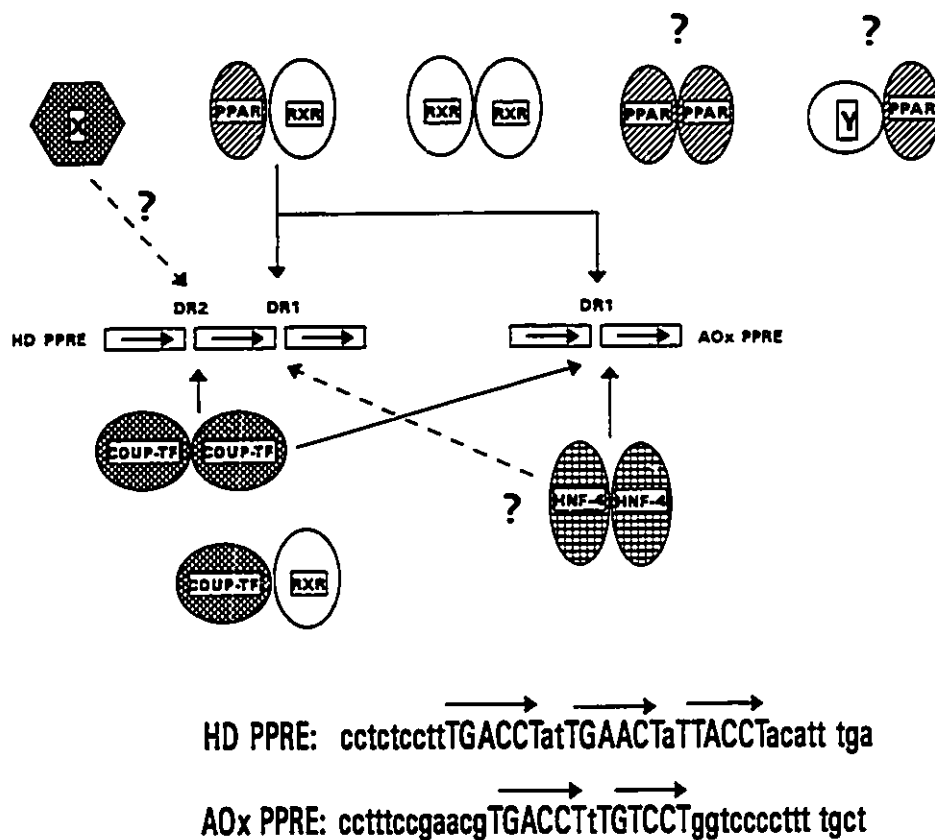


Fig. 3.29 Potential combinatorial interactions of nuclear proteins in the regulation of PPREs. The AOx PPRE consists of two direct repeats of the TGACCT motif with one nucleotide in between (DR1). The HD PPRE consists of three direct repeats of the consensus motif: the first and the second makes a DR2 while the second and the third makes a DR1. The PPAR/RXR dimer plays an important role by interacting with DR1 in both elements. HNF-4 can sufficiently bind to the AOx DR1 but only weakly to the HD DR1. The COUP-TF1 homodimerizes onto the DR1 in the AOx PPRE but interacts with the DR2 in HD PPRE. The later results in an antagonistic effect on the PPAR/RXR α -mediated transactivation. Additional unidentified factors are necessary for the PPREs to show maximal response to peroxisome proliferators. These factors could be HNF4, RXR α , PPAR in unknown multimerization (?) or novel factors X, Y.

to ciprofibrate. This leads to the conclusion that additional factors are involved in the positive regulation of the HD PPRE. Whether these additional factors are new trans-acting factors acting on their own as the X or as partners in a heterodimer such as Y/PPAR, will be interesting to investigate further (Fig. 3.29).

Such a combinatorial mechanism has in fact been a general rule for the expression of many inducible genes (Lucas and Granner, 1992). On one hand, multiple transcription factors that differ in their affinity and specificity for a given sequence yield an enormous combinatorial potential. On the other hand, protein-binding sequences in enhancers, promoters, and silencers neighbouring a gene, exist in multiple combinations and they function in response to diverse signals so that genes are regulated individually. The HD PPRE is interesting as it is a good example of these basic biochemical phenomena. It is only natural to expect progress in revealing the signal transduction pathway from peroxisome proliferator to the formation of PPAR/RXR α dimerization in the nucleus. New transcription factors and new PPREs will be found to reveal the mechanisms for a full induction of the peroxisomal genes. This signaling pathway would merge with hormonal signaling pathways. The induction mechanisms of peroxisomal genes would thus provide insight to the rules that govern the transcriptional machinery.

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