WestVirginiaUniversity
THE RESEARCH REPOSITORY @ WVU

Graduate Theses, Dissertations, and Problem Reports

2001

The role of accessory proteins in controlling the thyroid hormone regulation of transcription of the malic enzyme gene.

Yutong Wang

Follow this and additional works at: https://researchrepository.wvu.edu/etd

Recommended Citation

Wang, Yutong, "The role of accessory proteins in controlling the thyroid hormone regulation of transcription of the malic enzyme gene." (2001). *Graduate Theses, Dissertations, and Problem Reports*. 9968.
https://researchrepository.wvu.edu/etd/9968

This Thesis is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Thesis has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.

The Role of Accessory Proteins in Controlling the Thyroid Hormone Regulation of Transcription of the Malic Enzyme Gene

Yutong Wang

Dissertation submitted to the
School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
In
Biochemistry

F. Bradley Hillgartner, Ph.D., Chair
Marilyn I. Evans, Ph.D.
Stephen G. Graber, Ph.D.
Peter H. Mathers, Ph.D.
Jeannine S. Strobl, Ph.D.

Department of Biochemistry
Morgantown, West Virginia
2001

keywords: fatty acid synthesis, lipogenesis, gene expression, Pbx, chicken ovalbumin upstream transcription factor, CCAAT/enhancer-binding protein

UMI Number: 3014968

UMI Microform 3014968
Copyright 2001 by Bell & Howell Information and Learning Company.
All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

Bell & Howell Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

The Role of Accessory Proteins in Controlling the Thyroid Hormone Regulation of Transcription of the Malic Enzyme Gene

Yutong Wang

Triiodothyronine (T3) stimulates a 70-fold or greater increase in transcription of the malic enzyme gene in chick embryo hepatocytes. Previous work has shown that the effects of T3 on malic enzyme transcription are mediated in large part by sequence elements located within a liver-specific and T3-inducible DNase I hypersensitive region in the 5'-flanking DNA of the malic enzyme gene. Within this DNase I hypersensitive region is a cluster of nuclear T3 receptor (TR) binding sites that together constitute a T3 response unit (T3RU). Flanking the T3RU are accessory elements that enhance T3 regulation conferred by the T3RU in chick embryo hepatocytes. In this dissertation, I have characterized the proteins that bind TR accessory elements in the malic enzyme gene. I demonstrate that the homeodomain proteins, Pbx and Meis1, interact with a strongly active TR accessory element designated as region E. In addition, using a dominant-negative approach, I have developed functional evidence demonstrating that the liver-enriched transcription factor, CCAAT/Enhancer-binding protein (C/EBP), interacts with another TR accessory element referred to as region F. Results from transfection and protein-protein interaction assays suggest that the Pbx-Meis1 bound to region E and C/EBP bound to region F stimulate T3 responsiveness of the malic enzyme gene by directly interacting with TR bound to the T3RU. In further work, I have identified two new TR accessory elements that enhance T3 regulation of malic enzyme transcription in chick embryo hepatocytes. One element (designated as region G) binds chicken ovalbumin upstream promoter-transcription factor and the other element (designated as region H) binds an unidentified E-box binding protein. In summary, a wide variety of transcription factors mediate the potent effects of T3 on malic enzyme transcription in hepatocytes.

Acknowledgements

I would like to thank my advisor Dr. Brad Hillgartner for his encouragement, advice, and guidance during my graduate study. I would also express my deep appreciation by my graduate committee members, Dr. Marilyn Evans, Dr. Stephen Graber, Dr. Peter Mathers, and Dr. Jeannine Strobl for their valuable criticisms and suggestions on my research project. I would further extend my gratitude to all the Biochemistry Department faculty, staff and graduate students for their help and friendship.

I am thankful to Dr. Liya Yin for her help in glutathione S-transferase pull-down assay. I would also like to thank Dr. Yanqiao Zhang for his help in chick embryo hepatocytes and chick embryo fibroblasts culture.

Finally, I would like to dedicate this dissertation to my parents, and it is their love and support that made all these possible.

Table of Contents

List of Figures

List of Abbreviations

ARP1	apoAI regulatory protein 1
ATF-2	activating transcription factor-2
bHLH	basic-helix-loop-helix
bp	base pair
bZIP	basic region-leucine zipper
C/EBP	CCAAT/enhancer-binding protein
cAMP	adenosine 3',5'-cyclic monophosphate
CAT	chloramphenicol acetyltransferase
CBP	CREB binding protein
CEF	chick embryo fibroblasts
CEH	chick embryo hepatocytes
COUP-TF	chick ovalbumin upstream promoter transcription factor
CRE	cAMP response element
CREB	cAMP response element binding protein
cTRα	chicken nuclear T3 receptor α isoform
DBD	DNA binding domain
GST	glutathione S-transferase
GLUT4	glucose transporter 4
HD	homeodomain
HNF-4	hepatocyte nuclear factor-4
LBD	ligand binding domain
ME	malic enzyme
MEF2	myocyte-specific enhancer factor 2
Meis1	myeloid ecotropic viral integration site 1
N-CoR	nuclear receptor corepressor
NF-Y	nuclear factor-Y
Pbx	pre-B-cell leukemia transcription factor

PCE	Pbx-cooperativity element
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PPAR	peroxisome proliferator activated receptor
Prep1	Pbx-regulating protein 1
RAR	retinoic acid receptor
RSV	Rous sarcoma virus
RXR	retinoid X receptor
SMRT	silencing mediator for retinoid and thyroid hormone receptors
SRC	steroid receptor coactivator
SREBP	sterol regulatory element-binding protein
T3	3,3',5-triiodo-L-thyronine
T3RE	T3 response element
T3RU	T3 response unit
TALE	three amino acid loop extension
TBP	TATA box-binding protein
TF	transcription factor
TR	nuclear T3 receptor
TRAE	nuclear T3 receptor accessory element
USF	upstream stimulatory factor
uPA	urokinase plasminogen activator

Chapter 1. Literature Review

A. Introduction

Thyroid hormones are essential for cell growth, differentiation, and development in all vertebrates (Glass et al., 1990). In addition, thyroid hormones play an important role in the regulation of intermediary metabolism and thermogenesis. The active form of thyroid hormone, 3,5,3'-triiodothyronine (T3), exerts physiological effects by interacting with nuclear receptors that regulate transcription of specific genes. Alterations in the transcriptional activity of the nuclear T3 receptor are associated with abnormalities in the above processes and the development of disease. For example, a mutated version of the α form of the chicken nuclear T3 receptor, v-ErbA, participates in the neoplastic transformation of cells by the avian erythroblastosis virus (Gandrillon et al., 1987; Gandrillon et al., 1989; Graf et al., 1983). Unlike the wild type receptor, the v-*erb*A protein lacks the ability to bind T3 and activate transcription of genes normally induced by thyroid hormones (Muñoz et al., 1988; Privalsky et al., 1988). The aberrant transcriptional activity of the v-*erb*A protein may mediate its effects in neoplasia (Damm et al., 1989; Disela et al., 1991; Zenke et al., 1990). A role for altered nuclear T3 receptor function in the development of cancer in humans is indicated by the finding that several human lung carcinomas carry a deletion on chromosome 3 which overlaps with the TRβ gene (Dobrovic et al, 1988). Altered nuclear T3 receptor function is also associated with the development of obesity. In genetically obese (*ob/ob*) mice, the occupancy of the nuclear T3 receptor by ligand is decreased, and this may play a role in mediating reduced energy expenditure in these animals (Hillgartner et al, 1987). Some human obesities are characterized by decreases in nuclear T3 receptor levels in peripheral tissues (Moore et al, 1981). Another example of a link between altered T3 receptor function and disease is the finding that thyroid hormone resistance syndrome in humans is associated with mutations in the nuclear T3 receptor gene-β which decrease the ligand binding affinity and transcriptional activity of the encoded protein (Usala et al., 1991). Factors that modulate nuclear T3 receptor activity may prove to be potential targets in the treatment of cancer, obesity, and other thyroid hormone-related diseases in humans.

B. The Thyroid Hormone Receptor

Nuclear T3 receptors (TR) are cellular homologs of the v-*erb*A oncogene found in avian erythroblastosis virus (Sap et al., 1986; Weinberger et al., 1986). Different isoforms of the TR are expressed from two distinct genes, TRα and TRβ (Lazar et al., 1990). These isoforms are expressed tissue-specifically and in distinct patterns during development. The TRs are members of a superfamily of ligand-dependent transcription factors that includes the receptors for steroid hormones, vitamin A derivatives and vitamin D3 (Evans et al., 1988). TRs consist of 6 domains (A-F) based on amino acid sequence similarity with other members of the steroid receptor superfamily (Fig. 1). The DNA binding or "C" domain dictates target gene specificity. This highly conserved, cysteine-rich region is capable of forming two zinc finger DNA binding motifs that interact with half turns of the DNA. The DNA binding domain is necessary for nuclear localization of the receptor. Amino acid sequences encompassing a large area of the C-terminus (domains E and F) are required for ligand binding. Within domain E is a subdomain that mediates protein-protein interaction. This region consists of a series of nine hydrophobic heptad repeats which serve as an interface for dimer or oligomer formation. Domain E also contains a sequence that exerts a transcriptional activation function (TAF-2 or AF-2). The amino-terminal region of the receptor (domain A/B) also contains a sequence that has a transcriptional activation function (TAF-1 or AF-1). Amino acid sequences in the C-terminus of TR are also necessary for transcriptional repression in the absence of ligand (Baniahmad et al., 1992).

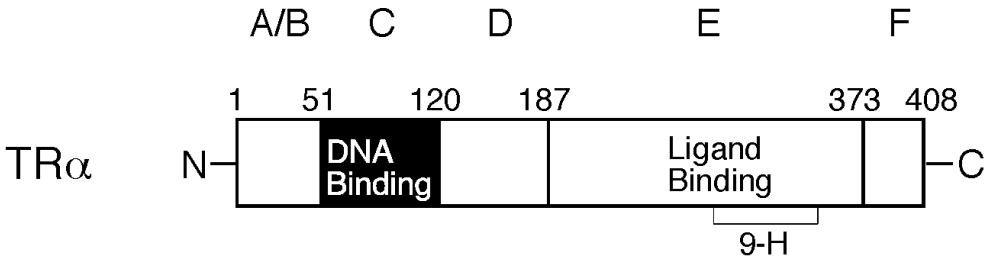

Figure 1. **Schematic representation of the primary structure of the chicken nuclear T3 receptor-α.**

A-F indicate the different domains of the protein based on amino acid sequence similarity with other members of the steroid hormone receptor superfamily. Regions C and E contain the conserved DNA binding domain (DBD) and ligand binding domain (LBD), respectively. Region A/B contains sequences that have a transcriptional activation function. D is the hinge region which contains a nuclear localization signal. F is a variable C-terminal region. Numbers above the receptor indicate amino acid boundaries of these domains, as well as the total amino acid length of the protein. 9-H indicates the position of the 9 hydrophobic heptad repeats which mediate protein-protein interactions.

TRs specifically bind to DNA sequences that confer T3-mediated regulation on promoter activity (Glass et al., 1990). These sequences are referred to as T3 response elements (T3RE). T3REs are typical enhancers, conferring T3-mediated regulation on adjacent genes in a manner independent of distance and orientation. In general, T3REs consist of multiple copies of a hexameric sequence related to a consensus (A/G)GGT(C/A)A arranged as direct or inverted repeats. Under most physiological conditions, TR is thought to bind to T3REs primarily as a heterodimer with the retinoid X receptor (RXR) (Yen and Chin, 1994). TR/RXR heterodimers bind to DNA with higher affinity and regulate transcription more effectively than TR homodimers or TR monomers (Kliewer et al., 1992; Leid et al., 1992; Yu et al., 1991). Other members of the nuclear hormone receptor superfamily, including the peroxisome proliferator activated receptor (PPAR) (Bogazzi et al., 1994), chick ovalbumin upstream promoter transcription factor (COUP-TF) (Cooney et al., 1992), and the all-trans retinoic acid receptor (RAR) (Glass et al., 1989) have been shown to heterodimerize with TR and modulate TR function. Based on functional transfection studies in T3-unresponsive cell lines, we (Hillgartner et al., 1992) and other laboratories (Damm et al., 1989; Yen and Chin, 1994) have shown that TRs exhibit at least two different activities. First, the unliganded form of TR is a repressor of transcription. Second, binding of T3 to the receptor relieves this repression. In some instances, binding of T3 causes a further activation of transcription above that observed in the absence of TR (Damm et al., 1989). Heterodimerization of TR with RXR enhances the both the repressor activity and the T3-dependent derepression/activation activity of the TR (Kliewer et al., 1992; Reginato et al., 1996) (Fig. 2). The repressor activity of the unliganded TR is dependent on physical interactions between TR and other nuclear proteins that lack intrinsic DNA binding activity. These proteins are referred to as corepressors and include N-CoR and SMRT (Chen and Evans, 1995; Horlein et al., 1995). In the absence of T3, corepressors may promote non-productive interactions between TR and the general transcriptional apparatus (Baniahmad et al., 1993; Fondell et al., 1993). Corepressors may also function by recruiting histone deacetylase-1 or 2 and mSin3A or -B to the promoter (Glass and Rosenfeld, 2000). The presence of a histone deacetylase activity in the corepressor complex may lead to local changes in the structure of the chromatin template, rendering it less accessible to the general transcriptional apparatus. In the presence of T3, the corepressor complex dissociates from TR, triggering the activation process (Glass and Rosenfeld, 2000). This may be followed or coincident with an induced

conformational change in the C-terminal activation domain of TR, allowing association with another complex of nuclear proteins that mediate the activation of transcription. These coactivator complexes lack intrinsic DNA binding activity. Many of the proteins comprising the coactivator complex have been identified; they include CREB binding protein (CBP) (Kamei et al., 1996), steroid receptor coactivator-1 (SRC-1) (Onate et al., 1995), and numerous other proteins (Edwards, 1999). Because several of these proteins possess histone acetyltransferase activity, the coactivator complex has been proposed to activate transcription by remodeling the chromatin template through targeted histone acetylation (Xu et al., 1999). This would facilitate the assembly of the general transcriptional apparatus on chromatin and stabilize interactions between the general transcriptional apparatus and the liganded TR/coactivator complex. While it is well accepted that corepressors and coactivators are important components of the T3 signaling pathway, evidence that these proteins play a role in mediating nutritional, developmental and tissue-specific regulation of T3 action is presently lacking.

In addition to the T3RE, cis-acting sequences that bind proteins that are distinct from TR/RXR play an important role in conferring T3 regulation on transcription. Such sequences are referred to as nuclear T3 receptor accessory elements (TRAEs). TRAEs in isolation are devoid of T3 responsive activity, but rather function to enhance the degree of T3 responsiveness 'initiated' by the T3RE. TRAEs have been identified in several thyroid hormone responsive genes. For example, in hepatoma cells, stimulation of phosphoenolpyruvate carboxykinase promoter by T3 is dependent on the presence of a T3RE and binding sites for the liver-enriched transcription factors CCAAT/enhancer binding protein-α and β (C/EBPα and C/EBPβ) (Park et al., 1995; Park et al., 1999). Schaufele and co-workers (Schaufele et al., 1992) have shown that T3 responsiveness of the rat growth hormone promoter in pituitary cells is dependent on a synergistic interaction between TR and the pituitary-specific DNA binding protein, Pit-1. In primary hepatocyte cultures, T3 stimulation of the S14 gene requires the presence of a nuclear factor-Y (NF-Y) binding site; this sequence is separated from the T3RE by greater than 2 kb (Jump et al., 1997). Recently, our laboratory has shown that T3-mediated regulation of malic enzyme transcription in primary hepatocyte cultures is enhanced by the presence of four TRAEs in the malic enzyme 5'-flanking region (Fang and Hillgartner, 1998). These cis-acting elements mediate most of the differences in T3 responsiveness of the malic enzyme gene between hepatocytes and non-hepatic cells. Collectively, the above findings suggest that a wide variety of

DNA-bound transcription factors can functionally interact with TR to modulate the transcriptional effects of T3. Presently, the mechanism(s) by which TRAE binding proteins modulate the transcriptional effects of TRs is not well known.

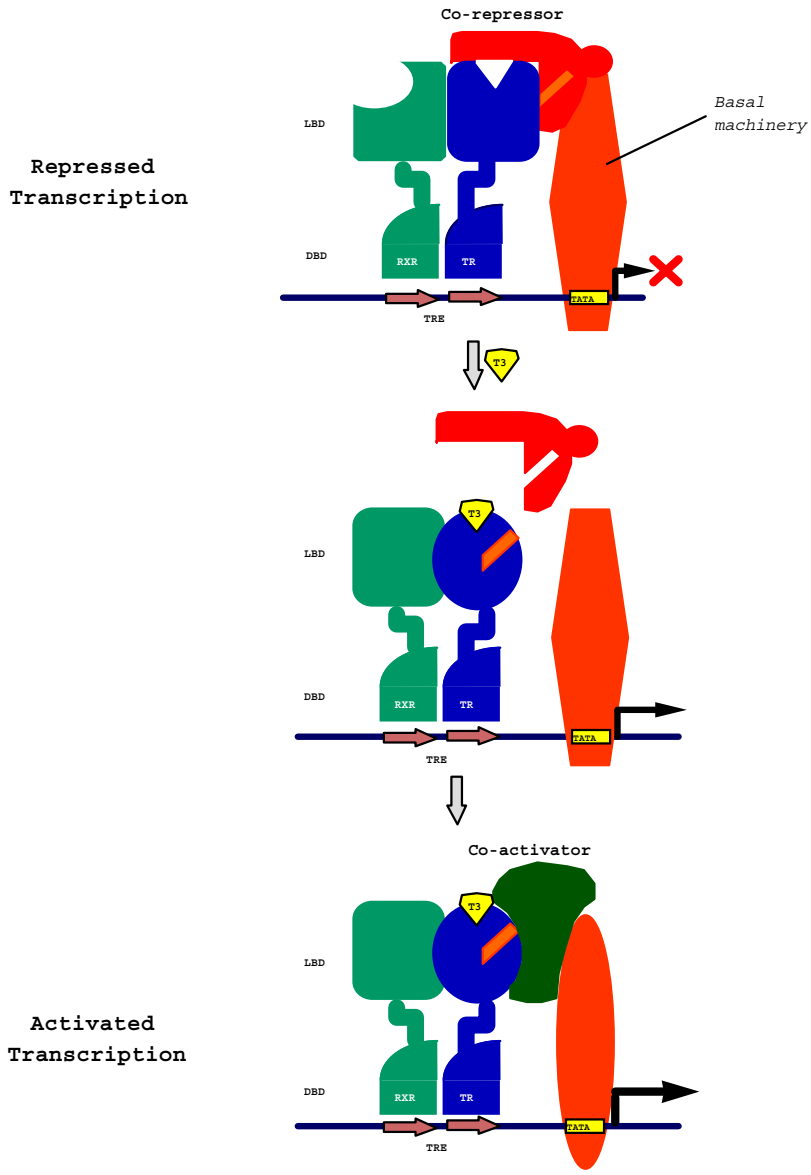

Figure 2. Model for thyroid hormone action.

In this model, the TR-RXR heterodimer is bound to a direct repeat T3 response element (T3RE) in the promoter of the target gene. In the absence of T3, the receptor complex communicates with the basal transcription initiation complex through a co-repressor and transcription is repressed. T3 binding induces a conformation change in the TR that displaces the co-repressor and exposes the activation domain of the receptor. The receptor complex then binds a co-activator which results in an activation of transcription above the basal level. LBD, ligand binding domain. DBD, DNA binding domain.

C. The Malic Enzyme Gene

1. Hormonal and Nutritional Regulation of Malic Enzyme Activity

Malic enzyme catalyzes the oxidative decarboxylation of malate to pyruvate and carbon dioxide, simultaneously generating NADPH from $NADP^+$. The malic enzyme reaction supplies about 50% of the NADPH required for palmitate synthesis in rat liver and adipose tissue and nearly all of the NADPH needed for palmitate synthesis in chicken liver (Goodridge, 1968; Hillgartner et al., 1995). Malic enzyme activity in liver are correlated with rates of fatty acid synthesis during different nutritional conditions. Thus, malic enzyme activity is increased during feeding a high-carbohydrate, low-fat diet and decreased during starvation or feeding a low-carbohydrate, high-fat diet (Goodridge, 1968).

Insulin, glucagon, triiodothyronine (T3) and glucose are thought to be the primary circulating factors that communicate changes in nutritional state to the liver, thereby mediating the effects of dietary manipulation on hepatic malic enzyme activity (Goodridge, 1987). This deduction is based on the observation that stimulation of malic enzyme activity caused by feeding a high-carbohydrate, low-fat diet is preceded or paralleled by increases in the molar ratio of insulin/glucagon and the levels of T3 and glucose in the blood. A major role for insulin, glucagon, T3, and glucose is also indicated by the observation that these agents regulate expression of lipogenic enzymes in various cell culture systems. For example, in primary cultures of chick embryo hepatocytes, T3 stimulates a 20-fold increase in malic enzyme activity (Goodridge and Adelman, 1976; Hillgartner and Charron, 1998). Glucose (20 mM) has no effect by itself on malic enzyme activity but amplifies the effect of T3 by two-fold. Insulin accelerates the increase in malic enzyme activity caused by T3 and glucose, whereas, glucagon inhibits the stimulatory effect of T3 and glucose by 95%.

Based on immunochemical studies, alterations in malic enzyme activity caused by starvation and refeeding in intact chickens and by insulin, glucagon, T3, and glucose in chick embryo hepatocytes are accounted for by changes in the concentration of malic enzyme which, in turn, are mediated by changes in the synthesis rate of malic enzyme. Nutrient- and hormone-induced changes in malic enzyme synthesis are accompanied by similar alterations in the abundance of malic enzyme mRNA. Further analyses have shown that the regulation of the

abundance of the malic enzyme mRNA by nutrients and hormones is mediated primarily by a transcriptional mechanism (Ma et al., 1990; Salati et al., 1991; Hillgartner et al., 1996; Hillgartner and Charron, 1998).

Recently, Goodridge and co-workers have shown that the T3-induced increase in malic enzyme transcription in chick embryo hepatoctyes is mediated by several T3REs in the 5'-flanking region of the malic enzyme gene (Hodnett et al., 1996; Thurmond and Goodridge, 1998). One strongly active site and four weakly active sites are located between -3889 and -3769 bp relative to the transcription start site (Fig. 3). This 120 bp region is referred to as a T3 response unit (T3RU). Another weakly active T3RE is located 800 bp downstream of the T3RU and is referred to as T3RE-7. Each of these T3REs resembles direct repeat elements with 4 bp spacing and bind RXR/TR heterodimers in nuclear extracts from chick embryo hepatocytes. The T3RU overlaps with a region of DNase I hypersensitivity in chromatin of chick embryo hepatocytes. This hypersensitive site is referred to as region IV (Fang and Hillgartner, 1998). Interestingly, the intensity of region IV increases when chick embryo hepatocytes are incubated with T3. The downstream T3RE overlaps with another region of DNase I hypersensitivity that is referred to as region III. In contrast to region IV, T3 treatment has no effect on the intensity of region III. DNase I hypersensitivity in region III and region IV is not observed in chromatin of non-hepatic tissues (i.e. heart and kidney) (Ma et al., 1990) or cell lines (i.e. chick embryo fibroblasts) (Fang and Hillgartner, 1998). DNase I hypersensitive sites are thought to represent regions of chromatin that have undergone changes in tertiary structure as a result of protein-DNA interactions involved in the control of transcription initiation.

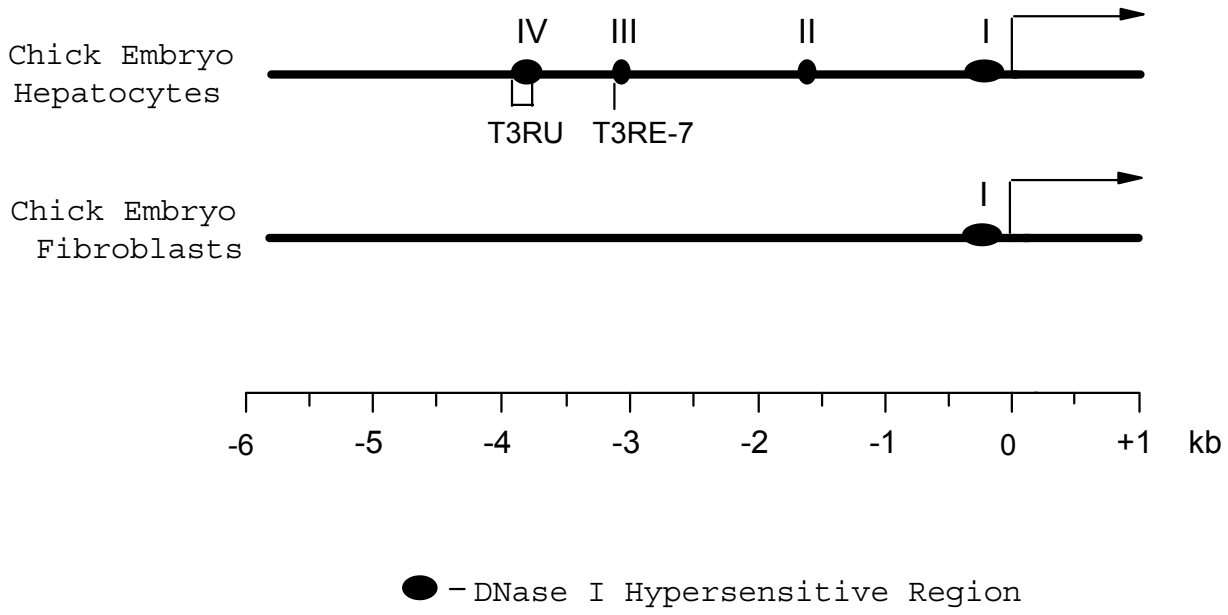

Figure 3. Schematic representation of the 5'-flanking region of the malic enzyme gene.

The major transcription start site is indicated by an arrow. DNase I hypersensitive regions are indicated by solid ovals. Hypersensitive region IV co-localizes with a cluster of five T3 response elements that is referred to as a T3 response unit (T3RU). Hypersensitive region III co-localizes with a single T3RE that is referred to as T3RE-7. The significance of hypersensitive region II is presently unclear as no cis-acting regulatory sequences have been identified in this region. Regions II, III, and IV are detected in chromatin of chick embryo hepatocytes but not in chromatin of chick embryo fibroblasts. Hypersensitive region I is located in the proximal promoter region and is observed in chromatin of both chick embryo hepatocytes and chick embryo fibroblasts.

2. Cell-Type Dependent Regulation of the Malic Enzyme Gene

An interesting feature of the malic enzyme gene that distinguishes it from other models of gene regulation is that its expression is observed in most tissues and cell lines, but its regulation by T3 and nutritional state is restricted to hepatic cells (Fang and Hillgartner, 1998). For example, malic enzyme transcription in heart, kidney, and brain is unresponsive to nutritional manipulation (Ma et al., 1990). In addition, T3 has no effect on malic enzyme transcription in avian fibroblast cell lines (Hillgartner et al., 1992). Our laboratory has shown that diminished T3 responsiveness of malic enzyme transcription in chick embryo fibroblasts relative to chick embryo hepatocytes is due to a decrease in the specific transcriptional activity of the nuclear T3 receptor in the former cell type. This is based on the observation that nuclear T3 receptor levels are similar in chick embryo hepatocytes and chick embryo fibroblasts (Hillgartner et al., 1992). Furthermore, in chick embryo fibroblasts that stably overexpress the α-isoform of chicken nuclear T3 receptor, stimulation of malic enzyme transcription by T3 is only 8% of that observed in chick embryo hepatocytes.

Using DNase I hypersensitivity, functional transfection, and *in vitro* DNA-binding analyses, our laboratory has identified four cis-acting elements in the malic enzyme 5'-flanking DNA that confer differences in nuclear T3 receptor activity between chick embryo hepatocytes and chick embryo fibroblasts (Fang and Hillgartner, 1998). These cell-type specific regulatory elements are located at -3895/-3890, -3761/-3744, -3703/-3686, and -3474/-2715 bp and are designated as region A, region E, region F, and region I, respectively. Each element enhances T3 responsiveness of the malic enzyme promoter in chick embryo hepatocytes but has no effect on T3 responsiveness in chick embryo fibroblasts. Three of the cell-specific regulatory elements (i.e. regions A, E, and F) flank the T3RU and overlap with a portion of DNase I hypersensitive region IV observed in chromatin of chick embryo hepatocytes (Fig. 4). Based on the results of *in vitro* DNA binding analyses, region F binds to the liver-enriched factor, CCAAT/enhancer-binding protein, whereas, regions A and E bind proteins of unknown identity. While region E contains sequences that resemble binding sites for CCAAT/enhancer-binding protein, activator protein-1, cyclic AMP response element binding protein, and NF-1, oligonucleotide competition and antibody supershift analyses indicated that none of these proteins bind to this DNA fragment.

The nature of the factor(s) that binds to region E is of interest because mutation of this sequence element causes the largest decrease in T3 responsiveness (87%) of the three cell-specific regulatory elements identified in region IV. The proteins that bind region A also have not yet been defined.

In addition to region A, region E, and region F, other sequences flanking the malic enzyme T3RU are likely to be involved in modulating T3 action. This conjecture is based on results from *in vitro* DNase I footprinting analyses (Fang and Hillgartner, 1998) indicating that sequences between -3681 and -3666 bp (designated as region G), -3655 bp and –3646 bp (designated as region H) are weakly protected in chick embryo hepatocytes but not in chick embryo fibroblasts (Fig. 4). Functional evidence that these sequences confer enhanced T3 responsiveness on malic enzyme promoter activity in chick embryo hepatocytes remains to be developed.

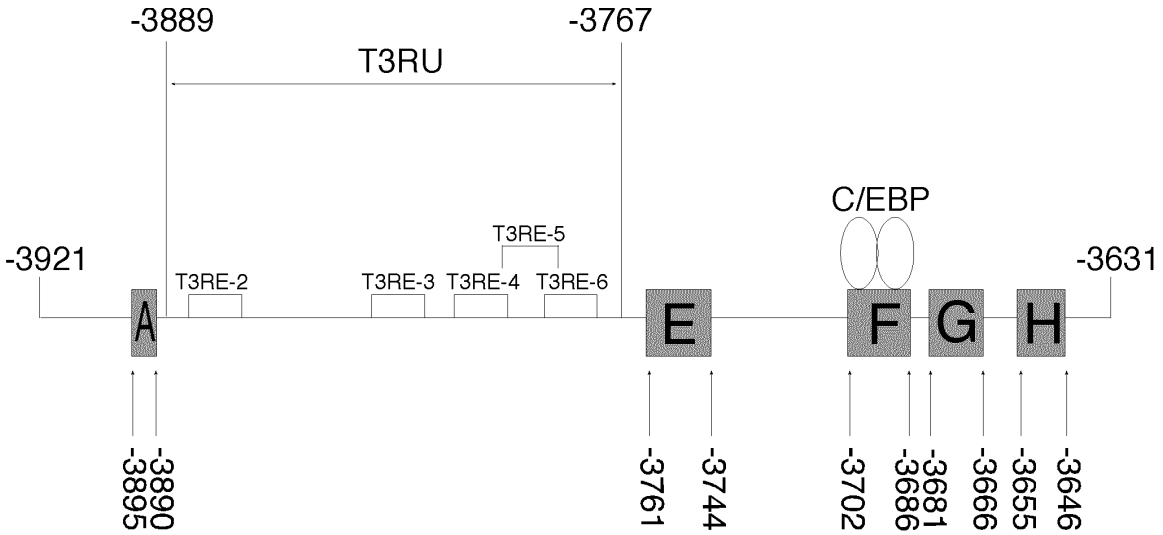

Figure 4. Schematic representation of region IV of the malic enzyme gene.

The locations of T3REs and TR accessory elements (i.e. regions A, E, and F) in DNase I hypersensitive region IV are indicated. Locations of putative TR accessory elements (i.e. regions G and H) are also indicated. The factor binding to region F, C/EBP, is illustrated. The subregion (-3889 to -3769 bp) containing the cluster of T3REs is referred to as the T3 response unit (T3RU).

D. Pbx

Pbx1, Pbx2 and Pbx3 are members of PBC subfamily of homeodomain proteins in vertebrate. Highly conserved homologs of Pbx have been identified in *Drosophila melanogaster*, *Caenorhabditis elegans*, and zebrafish (Burglin, 1997; Popperl et al, 2000). Pbx1 gene was discovered as a proto-oncogene which is translocated in pre-B acute lymphoblastoid leukemias (Kamps et al., 1990; Nourse et al., 1990). This gene codes for two different protein, Pbx1a and Pbx1b, due to alternative splicing (Monica et al., 1991).

Genetic and biochemical analyses have shown that Pbx proteins regulate developmental pathways by serving as cofactors for other homeodomain transcription factors. For example, Pbx potentiates the effects of Hox proteins on segmentation along the anterior-posterior axis (Lumsden and Krumlauf, 1996). This effect is mediated by heterodimeric interactions between Pbx and Hox. Pbx-Hox complexes bind DNA with enhanced specificity and affinity compared to complexes containing Pbx or Hox alone (Chang et al., 1995). Pbx also binds cooperatively to DNA with other TALE (three-amino acid loop extension) class homeodomain proteins including Meis1 and closely related factor, Prep1 (also known as pKnox1) (Berthelsen et al., 1998; Chang et al., 1997). Complexes containing Pbx-Meis1 or Pbx-Prep1 are also components of the transcriptional network controlling cell fate and segmental patterning during embryonic development (Lumsden and Krumlauf, 1996).

Recent work has shown that Pbx, Meis1, and Prep1 also function in maintaining cellular differentiation in adult tissues. For example, Pbx-Prep1 heterodimers mediate the pancreatic-specific transcription of the genes for somatostatin and glucagon (Goudet et al., 1999; Herzig et al., 2000). In the case of the somatostatin gene, Pbx-Prep1 stimulates transcription in pancreatic cells by directly interacting with and potentiating the transcriptional activity of the pancreatic-specific homeodomain factor, Pdx1 (Peers et al., 1995). In the case of the glucagon gene, Pbx-Prep1 binds to a cis-acting element that represses glucagon transcription in non-pancreatic cells without affecting glucagon transcription in pancreatic cells (Goudet et al., 1999). Other work has shown that a Pbx-Meis-Pdx1 trimeric complex activates transcription of the elastase I gene in pancreatic acinar cells via interactions with the acinar cell-specific factor, PTF1 (Swift et al., 1998). Still other studies have shown that Pbx-Prep1 heterodimers interact with a cis-acting

element that confers cell-type specific transcription of the human α2(V) collagen gene (Penkov et al., 2000). In addition to the pancreas and tissues that express α2(V) collagen, Pbx, Meis, and Prep1 proteins are expressed at significant levels in other adult tissues, including liver. The physiological role for Pbx, Meis, and Prep1 in liver has not been established.

Pbx and Meis1/Prep1 bind 4 bp sequence elements referred to as half-sites. Selection studies with degenerate oligonucleotides have shown that Pbx-Meis1 and Pbx-Prep1 heterodimers bind two contiguous half-sites with the consensus sequence, 5'-TGAT-TGAC-3', in which Pbx and Meis1/Prep1 contact the upstream and downstream half-sites, respectively (Knoepfler et al., 1997; Chang et al., 1997). This sequence is referred to as the PCE (Pbx-cooperativity element). Heterodimeric interaction between Pbx1 and Meis1/Prep1 can occur in solution and is mediated by isoleucine heptad repeats motifs in the N-termini of these proteins (Knoepfler et al., 1997).

E. CCAAT/Enhancer-Binding Protein

CCAAT/enhancer-binding protein (C/EBP) is a member of the basic region-leucine zipper (bZIP) class of transcription factors. The prototypic C/EBP is a modular protein, consisting of an activation domain, a DNA-binding basic region, and a dimerization domain (Agre et al., 1989; Vinson et al., 1993). The dimerization domain, termed as the "leucine zipper", is a heptad of leucine repeats that intercalate with repeats of the dimer partner, forming a coiled coil of α-helices in parallel orientation. Electrostatic interaction between the dimerization interfaces determines the specificity of dimer formation among C/EBP family members as well as with transcription factors of the NF-κB and Fos/Jun families (Vinson et al., 1993). The C/EBP family consists of at least six members, including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and CHOP which bind to specific DNA sequences as homo- or heterodimers (Yano et al., 1996). C/EBPα was originally purified and cloned as a liver-enriched factor (Johnson et al., 1987). It will bind to and activate transcription from several liver- and adipose-specific promoters including aP2-422 (Cheneval et al., 1991), phosphoenolpyruvate carboxykinase (PEPCK) (Park et al., 1990), glucose transporter 4 (GLUT4) (Kaestner et al., 1990) and C/EBPα

itself (Christy et al., 1991). Two isforms of C/EBPα are generated from its mRNA by a ribosomal scanning mechanism (Lin et al, 1993). The full-length protein is 42 kDa and contains three transactivation domains (TEI-III) (Nerlov and Ziff, 1994). TEI and TEII mediate cooperative binding of C/EBP to TATA box-binding protein (TBP) and TFIIB (Nerlov and Ziff, 1995). TFIII contains a negative regulatory subdomain which masks the activities of the transactivation domains and the DNA-binding bZIP domain.

C/EBPs are pivotal regulators of cellular differentiation, metabolic homeostasis, and inflammatory response. C/EBPα is the predominant nuclear signal regulating terminal hepatocyte differentiation and function. Elimination of C/EBPα in a mouse knockout model results in profound derangement of liver structure and function (Flodby et al., 1996; Wang et al., 1995). C/EBPα knockout mice exhibit disturbed hepatic architecture with acinar formation (Wang et al., 1995). Expression of c-Myc and c-Jun is induced in C/EBPα knockout mice, consistent with an inhibitory role of C/EBPα on liver cell proliferation (Flodby et al., 1996). C/EBPα knockout mice also exhibit pronounced metabolic derangement in hepatic carbohydrate and lipid metabolism, and the majority of mice die of hypoglycemia soon after birth (Wang et al., 1995).

F. Chicken Ovalbumin Upstream Promoter Transcription Factor

The chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are orphan members of the steroid/thyroid nuclear hormone receptor superfamily (Wang et al., 1989; Wang et al., 1991). There are no known ligands for COUP-TFs, and hence they have been assigned as a subclass of nuclear hormone receptors referred to as orphan receptors. Two COUP-TF genes, COUP-TFI AND COUP-TFII (also called apoAI regulatory protein-1, ARP-1) have been identified. The proteins encoded by these genes are closely related with an overall amino acid identity of 87% (Wang et al., 1989; Richie et al., 1990; Wang et al., 1991).

Originally, COUP-TFs were purified from HeLa cells as proteins capable of binding to an enhancer element in the chicken ovalbumin upstream promoter (Sagami et al., 1986). They are expressed ubiquitously and play a role in the regulation of several important biological processes, such as neurogenesis, cell fate determination, and carbohydrate metabolism (Kastner et al., 1995; Mangelsdorf and Evans, 1995). Several of the physiological actions of COUP-TFs are attributed to their ability of function as inhibitors of gene transcription (Tran et al., 1992; Kimura et al., 1993, Klinge et al., 1997). Several mechanisms account for the repressive effects of COUP-TFs. One mechanism involves forming nonproductive complexes with retinoid X receptor, the essential heterodimer partner of a number of nuclear hormone receptors (Cooney et al., 1993; Leng et al., 1996). Another mechanism involves competition by COUP-TF for binding to response elements of several nuclear hormone receptors including TR, peroxisome proliferator receptor, retinoic acid receptor, and vitamin D receptor (Pereira et al., 1995). COUP-TFs have also been shown to activate gene transcription by functioning as accessory factors for contiguously bound transcription factors (Sugiyama et al., 2000). In addition, COUP-TFs have been shown to function as coactivator proteins for some transcription factors such as hepatocyte nuclear factor-4 (Ktistaki and Talianidis, 1997). COUP-TFs can also activate transcription by specifically binding cis-acting elements of target genes and recruiting coactivators such as SRC-1 and SRC-2 (Stroup et al., 1997). The ability of COUP-TF to function as an activator and inhibitor of transcription may be due to the fact that the conformation of DNA-bound COUP-TF varies depending on the nature of the binding site. Previous studies have shown that DNA sequences can modulate transcription factor activity via an allosteric mechanism (Hwung et al., 1988; Cooney et al., 1992).

Chapter 2. The Homeodomain Proteins Pbx and Meis1 Are Accessory Factors That Enhance Thyroid Hormone Regulation of the Malic Enzyme Gene in Hepatocytes

A. Introduction

Malic enzyme catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2, simultaneously generating NADPH from $NADP^+$. This reaction is the primary source of reducing equivalents for *de novo* synthesis of long chain fatty acids in avian liver (Goodridge, 1968). Regulation of malic enzyme activity is typical of that of other lipogenic enzymes. Malic enzyme activity increases by 70-fold when previously starved chicks are fed a high-carbohydrate, low-fat diet and dramatically decreases when animals are starved (Goodridge, 1968). Nutritional regulation of malic enzyme activity is quantitatively mimicked in primary cultures of chick embryo hepatocytes by manipulating the concentrations of hormones and metabolic fuels in the culture medium. Insulin, glucose, and 3, 5, 3'-triiodothyronine (T3), humoral factors that are elevated during consumption of a high-carbohydrate, low-fat diet, increase malic enzyme activity in chick embryo hepatocytes (Goodridge et al., 1987). Glucagon and fatty acids, humoral factors that are elevated during starvation, inhibit the stimulation of malic enzyme activity caused by insulin, glucose and T3 (Goodridge et al., 1989). Alterations in malic enzyme activity caused by nutritional manipulation *in vivo* and nutrients and hormones in culture are mediated primarily by changes in the rate of transcription of the malic enzyme gene (Ma et al., 1990).

In addition to regulation by nutrients and hormones, malic enzyme transcription is controlled in a tissue-specific or cell-type-specific manner. In chicks fed a high-carbohydrate, low-fat diet, malic enzyme transcription is high in liver and low in heart, kidney and brain (Ma et al., 1990). Nutritional manipulation has no effect on malic enzyme transcription in heart, kidney, and brain. Cell-type dependent differences in the regulation of malic enzyme are also observed in cells in culture. T3 stimulates a 40-fold or greater increase in malic enzyme transcription in chick embryo hepatocytes, whereas in chick embryo fibroblasts and quail QT6 cells, T3 has little or no effect on malic enzyme transcription (Hillgartner et al., 1992).

The robust effects of T3 on malic enzyme transcription in chick embryo hepatocytes make this an excellent system to study the molecular mechanisms by which T3 regulates transcription. T3 activates gene transcription by interacting with nuclear T3 receptors (TR) (Evans, 1988). TRs bind T3 response elements (T3RE) of target genes as homodimers or heterodimers with the retinoid X receptor (RXR) (Hallenbeck et al., 1992; Yu et al., 1991; Kliewer et al., 1992). T3REs consist of multiple copies of a hexameric sequence related to a consensus RGGWMA arranged as inverted repeats, everted repeats, direct repeats or as extended single copies of the hexamer (Williams et al., 1991; Baniahmad et al., 1990; Näär et al., 1991; Farsetti et al., 1992). The T3-induced increase in malic enzyme transcription in chick embryo hepatocytes is mediated by at least six T3REs. One strongly active and four weakly active T3REs are clustered in a 114 bp region located at -3883/-3769 bp relative to the transcription start site (Hodnett et al., 1996; Thurmond and Goodridge, 1998). This cluster of T3REs is referred to as a T3 response unit (T3RU). Another weakly active T3RE is located about 700 bp downstream of the T3RU. All of the malic enzyme T3REs consist of directly repeated hexameric half-sites separated by a 4 bp spacer and bind TR-RXR heterodimers in hepatic nuclear extracts.

In addition to T3REs, accessory elements that bind nonreceptor proteins play an important role in mediating the effects of T3 on malic enzyme transcription. We have identified four accessory elements in the malic enzyme gene that confer differences in T3 regulation of malic enzyme transcription between chick embryo hepatocytes and chick embryo fibroblasts (Fang and Hillgartner, 1998). Each element enhances T3 responsiveness of the malic enzyme promoter in chick embryo hepatocytes but has no effect on T3 responsiveness in chick embryo fibroblasts. Three of the accessory elements immediately flank the T3RU and are designated regions A, E, and F. The T3RU and accessory elements A, E, and F overlap with a region of hepatocyte-specific DNase I hypersensitivity that is induced by T3 treatment. Region F (-3703/-3686 bp) binds the liver-enriched factor, CCAAT/enhancer-binding protein-α. The identities of the proteins that bind region A (-3895/-3890 bp) and region E (-3761/-3744 bp) have not yet been determined. The nature of the protein(s) that binds region E is of particular interest because this element stimulates the largest increase (> 4-fold) in T3-induced malic enzyme transcription of the four accessory elements identified.

Pbx proteins are members of the three amino acid loop extension (TALE) superclass of homeodomain proteins. The *Pbx1* gene was first identified as a site of t(1;19) chromosomal

translocation leading to the production of an E2a-Pbx1 fusion protein in a subset of pre-B cell acute lymphoblastic leukemias in humans (Kamps et al., 1990; Nourse et al., 1990). Highly conserved homologs of Pbx have been identified in *Drosophila melanogaster*, *Caenorhabditis elegans* (Burglin, 1997), and zebrafish (Popperl et al, 2000). Genetic and biochemical analyses have shown that Pbx proteins regulate developmental pathways by serving as cofactors for other homeodomain transcription factors. For example, Pbx potentiates the effects of Hox proteins on segmentation along the anterior-posterior axis (Lumsden and Krumlauf, 1996). This effect is mediated by heterodimeric interactions between Pbx and Hox (Chang et al., 1995). Pbx-Hox complexes bind DNA with enhanced specificity and affinity compared to complexes containing Pbx or Hox alone. Pbx also binds cooperatively to DNA with other TALE class homeodomain proteins including Meis1 and the closely related factor, Prep1 (also referred to as pKnox1) (Berthelsen et al., 1998; Chang et al, 1997). Complexes containing Pbx-Meis1 or Pbx-Prep1 are components of the transcriptional network controlling cell fate and segmental patterning during embryonic development (Ferretti et al., 1999).

Recent work has shown that Pbx, Meis1, and Prep1 also function in maintaining cellular differentiation in adult tissues. For example, Pbx-Prep1 heterodimers mediate the pancreatic-specific transcription of the genes for somatostatin and glucagon (Goudet et al., 1999; Herzig et al., 2000). In the case of the somatostatin gene, Pbx-Prep1 stimulates transcription in pancreatic cells by potentiating the transcriptional activity of the pancreatic-specific homeodomain factor, PDX1 (Goudet et al., 1999). In the case of the glucagon gene, Pbx-Prep1 binds to a cis-acting element that represses glucagon transcription in non-pancreatic cells without affecting glucagon transcription in pancreatic cells (Herzig et al., 2000). Other work has shown that a Pbx-Meis-PDX1 trimeric complex activates transcription of the elastase I gene in pancreatic acinar cells via interactions with the acinar cell-specific factor, PTF1 (Swift et al., 1998). Still other studies have shown that Pbx-Prep1 heterodimers interact with a cis-acting element that confers cell-type-specific transcription of the human $\alpha 2$(V) collagen gene (Penkov et al., 2000). In addition to the pancreas and tissues that express $\alpha 2$(V) collagen, Pbx, Meis, and Prep1 proteins are expressed at significant levels in other adult tissues, including liver. The physiological role for Pbx, Meis, and Prep1 in liver has not been established.

In this section, we have characterized the proteins that bind region E in hepatocyte nuclear extracts. We show that Pbx-Meis1 heterodimers bind region E in multiple

configurations. We also provide data suggesting that the stimulation of T3-induced malic enzyme transcription by region E is mediated by direct interactions between Pbx and TR.

B. Experimental Procedures

Plasmids - The DNA fragments used to construct reporter plasmids were named by designating the 5' and 3' ends of each fragment relative to the start site of transcription of the malic enzyme gene. p[ME-3921/-3631]ME-147/+31CAT has been previously described (Fang and Hillgartner, 1998). This reporter plasmid contains the malic enzyme T3RU and flanking sequences (-3921 to -3631 bp) linked to the minimal promoter of the malic enzyme gene (-147 to +31 bp). Site directed mutations were introduced into p[ME-3921/-3631]ME-147/+31CAT using a polymerase chain reaction-based strategy (Horton et al., 1990). p[T3RE2]ME-147/+31CAT was made by subcloning the major malic enzyme T3RE between -3883 to -3858 bp into the BamH I/Sac I site upstream of the minimal promoter in ME-147/+31CAT. p[T3RE2+E]ME-147/+31CAT was constructed by inserting region E (ME-3767/-3742) into the Spe I/Sma I site immediately downstream of the T3RE2 in p[T3RE2]ME-147/+31CAT. Dr. M. Kamps (University of California at San Diego) provided the cDNAs for human Pbx1a and Pbx1b. The cDNA for human Meis1 was obtained from Dr. N. Copeland (NCI-Frederick Cancer Research and Development Center). Drs. H. Chen and S. Antonarakis (University of Geneva) supplied the cDNA for human Prep1. cDNAs for full-length Pbx1a (Pbx1a 1-430), Pbx1b (Pbx1b 1-347), Meis1 (Meis1 1-390), and Prep1 (Prep1 1-436) and human RXRα (RXRα 1-462) were subcloned into pSV-SPORT1 (GIBCO/BRL). Expression plasmids containing N-terminal truncations of Pbx1a (Pbx1a 80-430 and Pbx1a 119-430) were provided by Dr. C. Murre (University of California at San Diego). The full-length cDNA of chicken TRα corresponding to amino acids 1 to 408 (TRα1-408) was subcloned into pGEM-3Zf(-) (Promega). N-terminal and C-terminal deletion derivatives of chicken TRα were generated by PCR. PCR products encoding TRα polypeptides containing amino acids 1-118, 1-157, 21-408, 51-408, and 120-408 were subcloned into pGEM-3Zf(-). To generate plasmids that express fusion proteins containing glutathione S-transferase (GST) linked to Pbx1a, Pbx1b or TRα, cDNAs containing the full-length coding region of Pbx1a, Pbx1b or TRα were subcloned into pGEX-2T (Amersham

Pharmacia). Structures of reporter plasmids and expression plasmids were confirmed by restriction enzyme mapping and nucleotide sequence analyses.

Cell Culture and Transient Transfection - Primary cultures of chick embryo hepatocytes were prepared as previously described (Goodridge, 1973; Fang and Hillgartner, 1998) and maintained in serum-free Waymouth medium MD705/1 containing 50 nM insulin (gift from Eli Lilly Corp.) and corticosterone (1 μM). Hepatocytes were incubated at 40°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were transfected using a modification of the method of Baillie et al. (Baillie et al., 1993). Briefly, chick embryo hepatocytes were isolated as described above and plated on 35 mm dishes. At 18 h of incubation, the medium was replaced with one containing 20 μg LipofectACE (GIBCO/BRL), 3.0 μg of p[ME-3921/-3631]ME-147/+31CAT or an equimolar amount of another reporter plasmid and 0.05 μg of pCMV-β-galactosidase. At 42 h of incubation, the transfection medium was replaced with fresh medium with or without T3 (1.5 μM). At 90 h of incubation, CEH were harvested and cell extracts were prepared for CAT (Gorman et al., 1982) and β-galactosidase (Sambrook et al., 1989) measurements. CAT activity was expressed relative to β-galactosidase activity to correct for differences in transfection efficiency between samples. All DNAs used in transfection experiments were purified using the Qiagen endotoxin-free kit.

Gel Mobility Shift Analysis - Nuclear extracts were prepared from chick embryo hepatocytes incubated with or without T3 for 24 h. Nuclei and nuclear extracts were prepared as described (Dignam et al., 1983) except that the protease inhibitors, leupeptin (0.25 μg/ml), benzamidine (10 mM), and PMSF (0.5 mM) were added to the extraction and dialysis buffers at the indicated concentrations. Pbx1a, Pbx1b, Meis1, Prep1, RXRα, and TRα were translated *in vitro* using the TNT SP6 coupled reticulocyte lysate system (Promega). Incorporation of ^{35}S-methionine into Pbx1a, Pbx1b, Meis1, Prep1, RXRα, and TRα was measured in parallel reactions in order to assess the relative efficiency of synthesis of the different transcription factors.

Double-stranded oligonucleotides were prepared by combining equal amounts of the complementary single-stranded DNA in a solution containing 10 mM Tris, pH 8.0, 1 mM EDTA followed by heating to 95°C for 2 min, and then cooling to room temperature. The annealed oligonucleotides were labeled by filling in overhanging 5' ends using the Klenow fragment of *E*.

coli DNA polymerase in the presence of [α-^{32}P]dCTP. Binding reactions were carried out in 20 µl containing 18 mM HEPES, pH 7.9, 90 mM KCl, 0.18 mM EDTA, 0.45 mM DTT, 18% glycerol (v/v), 0.3 mg/ml bovine serum albumin, and 2 µg of poly(dI/dC). A typical reaction contained 20,000 cpm of labeled DNA and 10 µg of nuclear extract or 2 µg of *in vitro* translated proteins. The reaction was carried out on ice for 60 min. DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 4°C in 0.5 X TBE (45 mM Tris, pH, 45 mM boric acid, 1 mM EDTA). Following electrophoresis, the gels were dried and subject to storage phosphor autoradiography. For competition experiments, unlabeled competitor DNA was mixed with radiolabeled oligomer prior to addition of nuclear extract. For gel supershift experiments, nuclear extracts or *in vitro* translated proteins were incubated with antibodies for 1 h at 0°C prior to addition of the oligonucleotide probe. An antiserum that reacts with all isoforms of Pbx (Pbx1, 2, and 3) (Monica et al., 1991) was generously provided by Dr. M. Kamps (University of California at San Diego). A polyclonal antibody that reacts with Meis1 and Prep1 (sc-6245) was purchased from Santa Cruz Biotechnology. Synthetic oligonucleotides that were used as probes or competitors in gel mobility shift assays are listed in Fig. 7A.

Protein-protein interactions - GST or GST fusion proteins were expressed in *E. coli.* (BL21, pLysS) and purified using standard techniques (Smith and Johnson, 1988). Briefly, bacteria were transformed with pGEX-2T or recombinant pGEX-2T plasmids expressing GST fusion proteins. Overnight bacterial cultures in ampicillin (250 µg/ml) were diluted 1:100 into 250 ml of Luria broth and grown at 37°C to an $O.D._{600} = 1.0$ before induction with 1 mM isopropylthiogalactopyranoside (IPTG) for 60 min. Cells were pelleted and resuspended in 5 ml of buffer A (50 mM KCl, 25 mM HEPES [pH 7.9], 6% glycerol, 5 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Triton X-100). Cells were lysed on ice by sonication and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was mixed for 1 h at 4°C on a rotator with 0.5 to 1 ml of 50% glutathione-sepharose beads (Amersham Pharmacia) which were preswollen in buffer A. After absorption, beads were collected by centrifugation at 4°C and washed three times with 1 ml of buffer A. Fusion proteins coupled to the glutathione-sepharose beads were stored at 4°C as 50% (v/v) slurry in buffer A. The concentrations and sizes of GST and GST fusion proteins were estimated by SDS-PAGE, using a known quantity of molecular weight standards.

L-[^{35}S]cysteine- or L-[^{35}S]methionine-labeled proteins were prepared by using TNT reticulocyte lysates (Promega). Approximately 2.5 x 10^4 to 5 x 10^4 cpm of ^{35}S-labeled protein was incubated with 100 ng of GST fusion protein immobilized on glutathione-sepharose beads in 300 µl of buffer A for 1 h at 4°C on a rotator. Beads were collected by centrifugation at 4°C and washed three times with 1 ml of buffer A. The bound proteins were eluted with SDS-gel loading buffer and analyzed by SDS-PAGE followed by storage phosphor autoradiography. Where indicated, 1 µM T3 was included in the binding reaction mixture. ^{35}S-labeled proteins were analyzed by electrophoresis and storage phosphor autoradiography to ensure that equal amounts of input radioactivity of the labeled protein were used in the GST binding assays.

C. Results

Pbx/Meis1 heterodimers bind region E of the malic enzyme gene.

In fully differentiated cells, Pbx has been shown to bind DNA as heterodimers with other homeodomain factors such as Meis1 and Prep1. Pbx and Meis1/Prep1 in heterodimeric complexes bind to separate 4 bp sequence elements referred to as half-sites. Selection studies with degenerate oligonucleotides have shown that Pbx-Meis1 and Pbx-Prep1 heterodimers bind two contiguous half-sites with the consensus sequence, 5'-TGAT-TGAC-3' in which Pbx and Meis1/Prep1 contact the upstream and downstream half-sites, respectively (Fig. 5A) (Knoepfler et al., 1997; Chang et al., 1997). This sequence is referred to as the PCE (Pbx-cooperativity element). Sequence comparison analysis of region E indicated the presence of four tetrameric sequence elements that are identical or strongly resemble binding sites for Pbx, Meis1, and Prep1. These half-sites are designated 1, 2, 3, and 4. To determine whether Pbx heterodimers interacted with region E, we analyzed the ability of the PCE to compete for protein binding to region E in gel mobility shift assays. Incubation of a ^{32}P-labeled oligonucleotide probe containing region E with nuclear extract from chick embryo hepatocytes resulted in the formation of two DNA-protein complexes that were designated a and b in the order of increasing mobility (Fig. 5B). An unlabeled oligonucleotide containing the PCE was more effective in competing for the binding of complex a and complex b than an unlabeled oligonucleotide containing region E. This observation suggests that heterodimers comprised of Pbx-Meis1 and/or Pbx-Prep1 bind

region E in hepatocytes. To obtain additional data demonstrating that Pbx-Meis1 or Pbx-Prep1 bind region E, antibody supershift experiments were performed. Incubation of hepatocyte nuclear extracts with antiserum against all isoforms of Pbx completely disrupted the formation of complex a and b indicating that these complexes contain Pbx or a highly related factor (Fig. 5C). Incubation of nuclear extracts with an antibody that reacts with Meis1 and Prep1 also completely disrupted the formation of complex a and complex b. Thus, complex a and complex b contain Pbx-Meis1 and/or Pbx-Prep1 heterodimers.

The ability of region E to bind heterodimers containing Pbx-Meis1 and Pbx-Prep1 was confirmed by gel mobility shift experiments employing *in vitro* synthesized transcription factors. At least 5 isoforms of Pbx have been identified that are derived from three different genes (Monica et al., 1991). Pbx1a, Pbx2, and Pbx3a are products of the *Pbx1*, *Pbx2*, and *Pbx3* genes, respectively. Pbx1b and Pbx3b are variants that arise from alternative processing. As Pbx1a and Pbx1b are expressed in liver, we investigated whether these proteins bind region E in the absence or presence of Meis1 and Prep1. Incubation of *in vitro* translated Pbx1a, Pbx1b, Meis1 or Prep1 with the region E probe resulted in no specific binding activity (Fig. 6). Inclusion of Meis1 in the binding reactions with Pbx1a and Pbx1b stimulated the formation of high-affinity protein-DNA complexes containing Pbx1a-Meis1 and Pbx1b-Meis1, respectively. Inclusion of Prep1 in the binding reactions with Pbx1a and Pbx1b also stimulated the formation of high-affinity heterodimeric complexes. Thus, Pbx1a and Pbx1b cooperatively interact with Meis1 or Prep1 to bind region E. Cooperative interactions between Pbx1 and Meis1 and between Pbx1 and Prep1 have been reported for other Pbx binding sites (Berthelsen et al., 1998; Chang et al., 1997). The mobilities of region E complexes containing Pbx1a-Prep1 and Pbx1b-Prep1 were slower than the mobilities of corresponding complexes containing Pbx1a-Meis1 and Pbx1b-Meis1. This was due to the larger size of Prep1 (436 amino acids) relative to Meis1 (390 amino acids). Interestingly, the mobility of complex a in hepatocyte nuclear extracts was identical to that of Pbx1a-Meis1 and the mobility of complex b was identical to that of Pbx1b-Meis1. This observation suggests that complex a and complex b contain Pbx1a-Meis1 and Pbx1b-Meis1, respectively. Complex a may also contain Pbx2-Meis1 heterodimers, as Pbx2 is approximately the same size as Pbx1a, is expressed in liver, and cooperatively binds DNA with Meis1 (Monica et al., 1991). Because expression of Pbx3a and Pbx3b in liver is very low compared to that of Pbx1 and Pbx2 (Monica et al., 1991), the former proteins are not likely to be present in complex a and complex b.

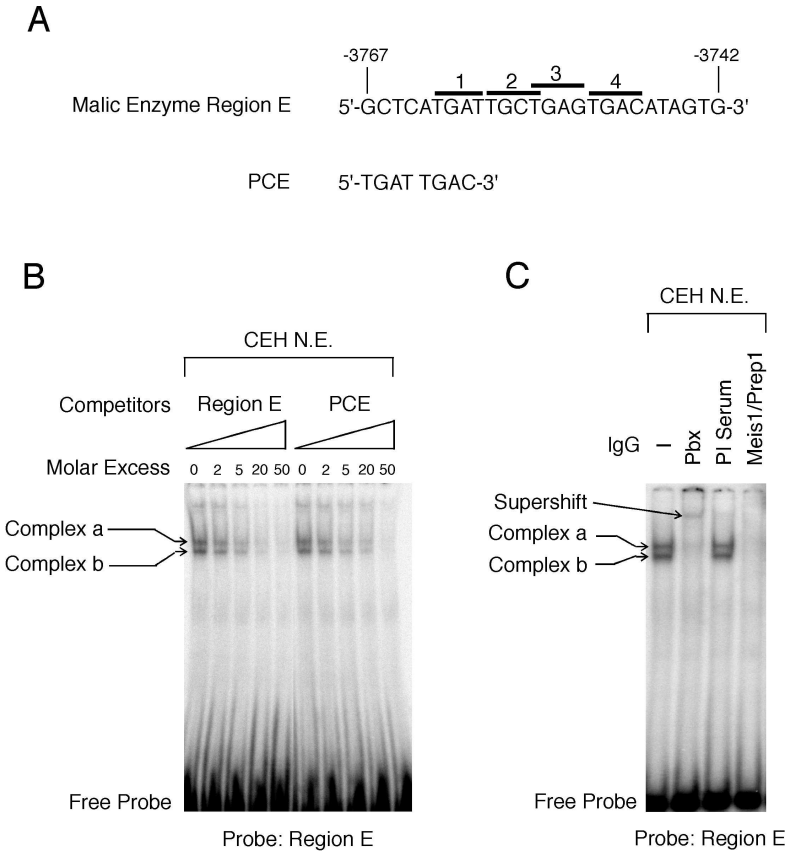

Figure 5. Characterization of proteins that bind to region E using the gel mobility shift assay.

(A) Comparison of the sequence of malic enzyme region E with Pbx-cooperativity sequence (PCE). The four potential tetrameric half sites are overlined. (B) Gel mobility shift experiments were performed using nuclear extracts prepared from chick embryo hepatocytes (CEH N.E.). A double-stranded DNA fragment containing region E (-3767 to -3742 bp) was labeled with [α-32]dCTP using the Klenow fragment of *E. coli* DNA polymerase. The radiolabeled probe was incubated with nuclear extract as described under "Experimental Procedures". DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels. Competition analysis was performed by mixing the labeled probe with a 2-, 5-, 20-, or 50-fold molar excess of unlabeled competitor DNA prior to the addition of the nuclear extract. (C) Nuclear extracts were incubated with antibodies against Pbx and Meis1/Prep1 prior to the addition of the probe. Positions of the specific protein-DNA complexes and supershifted complexes are indicated by arrows. These data are representative of three different experiments employing independent preparations of nuclear extract. Chick embryo hepatocyte nuclear extract, CEH N.E. Preimmune serum, PI serum.

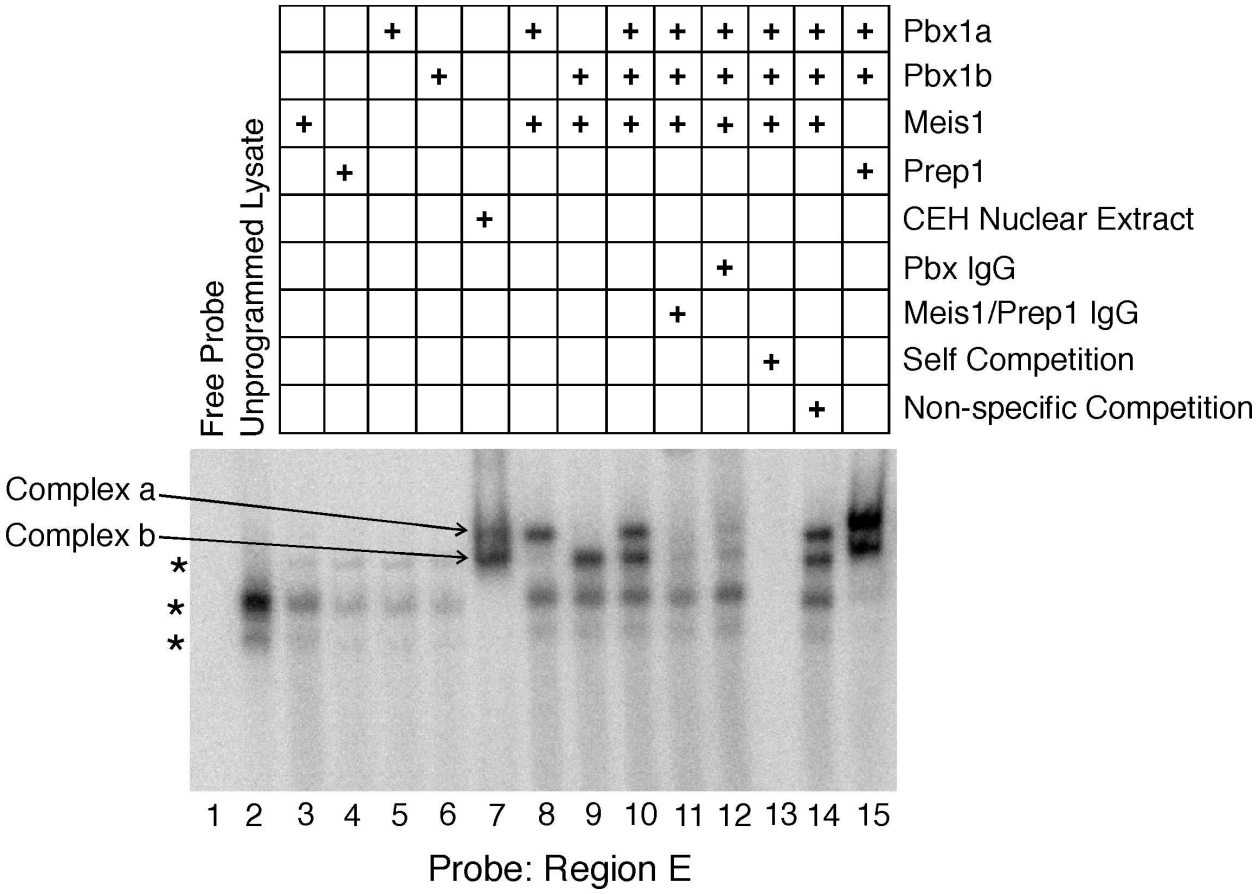

Figure 6. Pbx1 cooperatively interacts with Meis1 or Prep1 in binding region E.

Gel mobility shift assays were performed as described under "Experimental Procedures" using ^{32}P-labeled region E (-3767 to -3742 bp) as the probe. In lanes 3 to 6 and lanes 8 to 15, equimolar amounts of *in vitro* synthesized Meis1 or Prep1 were incubated in the absence or presence of *in vitro* synthesized Pbx1a or Pbx1b as indicated. In lane 7, the ^{32}P-labeled probe was incubated with nuclear extract from chick embryo hepatocytes. In lanes 13, competition analysis was performed with a 100-fold molar excess of unlabeled competitor DNA. In lanes 11 and 12, *in vitro* synthesized proteins were incubated with antibodies against Pbx or Meis1/Prep1 prior to the addition of the probe. Positions of heterodimeric complexes are indicated by arrows. Nonspecific complexes are indicated by an asterisk.

Identification of sequences in region E that bind Pbx/Meis1 heterodimers in nuclear extracts from hepatocytes.

In all of the native Pbx-Meis1 target genes identified to date, Pbx-Meis1 interact with cis-acting elements that consist of tandem 4 bp half-sites with no spacer between the half-sites (Chang et al., 1997). Studies with artificial sequence elements have shown that Pbx1-Meis1 also bind tandem half-sites that are separated by 3 or 6 nucleotides (Jacobs et al., 1999). The sequence in region E contains five possible binding configurations for Pbx-Meis1 heterodimers (Fig. 7A). Two of the binding configurations are comprised of two half-sites with no spacer (i.e. sites 1 and 2 and sites 3 and 4), another two binding configurations are comprised of two half-sites separated by a 3 bp spacer (i.e. sites 1 and 3 and sites 2 and 4), and one binding configuration contains two half-sites separated by a 7 bp spacer (i.e. sites 1 and 4). To determine which of these configurations bind complex a and complex b, competition experiments were performed with oligonucleotide competitors containing mutations in specific half-sites. Mutation of site 1 (mut E 1), site 2 (mut E 2), or site 3 (mut E 3) had little or no effect on the binding affinity of complex a and complex b, whereas, mutation of site 4 (mut E 4) decreased the binding affinity of complex a and complex b (Fig. 7B). These results suggest that complex a and complex b bind region E in two or more configurations involving site 4. To further delineate the region E binding configurations, competition analyses were conducted with oligonucleotides containing specific mutations in two of the four half-sites. The combination of sites 2 and 4 (mut E 1/3), sites 2 and 3 (mut E 1/4), and sites 1 and 3 (mut E 2/4) abolished protein binding to region E. Combination of sites 1 and 2 (mut E 3/4) inhibited but did not abolish the binding of complex a and complex b to region E. The combination of sites 3 and 4 (mut E 1/2) or sites 1 and 4 (mut E 2/3) had protein binding activities similar to that of the wild type sequence. Collectively, these data indicate that complex a and complex b bind region E in the following configurations: half-sites 1 and 2, half-sites 1 and 4, and half-sites 3 and 4. The binding affinity of complex a and complex b for half-sites 1 and 2 is lower than that for half-sites 1 and 4 and half-sites 3 and 4. Competition analysis with region E mutants indicated that *in vitr*o synthesized Pbx1a-Meis1 and Pbx1b-Meis1 bound region E in a manner identical to that of complex a and complex b (data not shown). These data are consistent with the model that complex a and complex b contain Pbx1a-Meis1 and Pbx1b-Meis1, respectively.

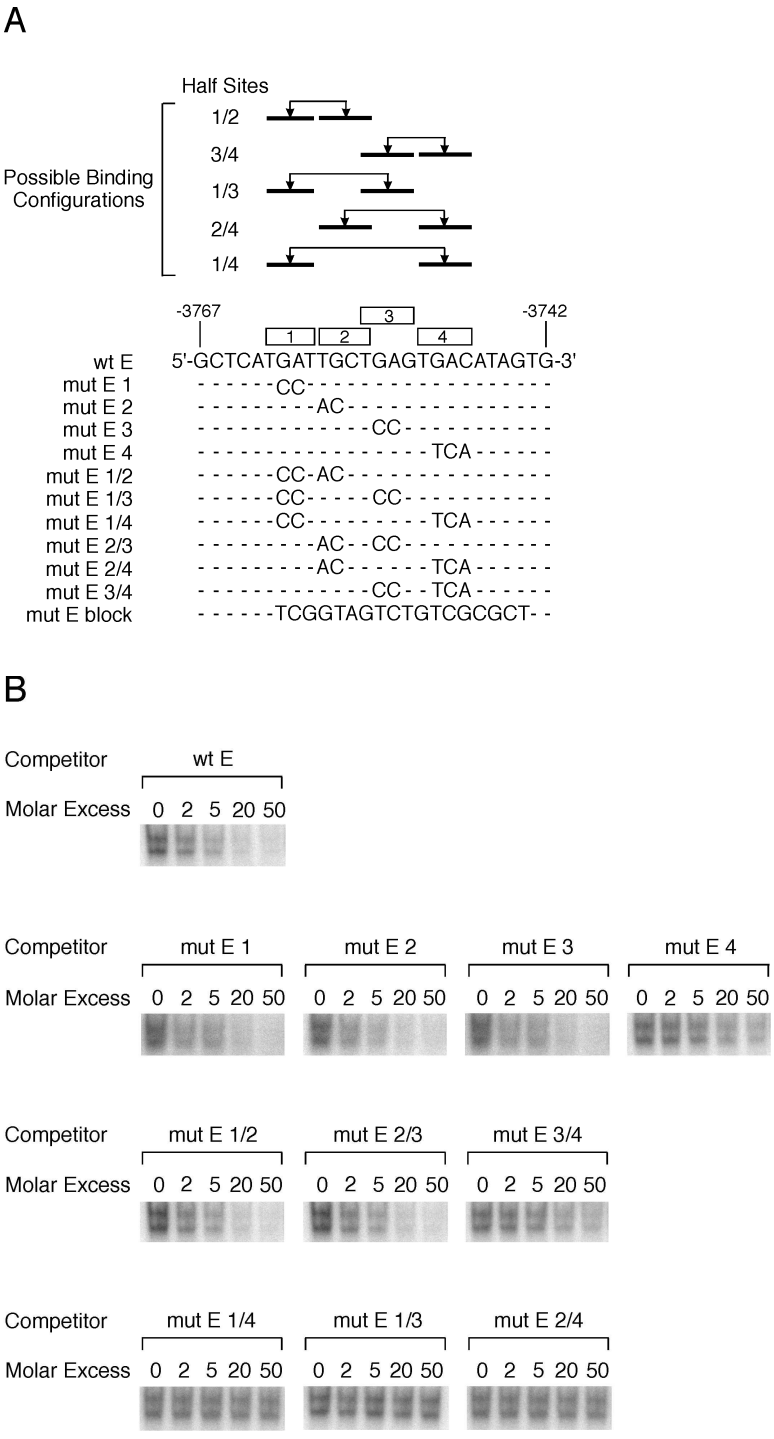

Figure 7. Complex a and complex b bind region E in multiple configurations.

Gel mobility shift experiments were performed as described under "Experimental Procedures" using ^{32}P-labeled region E (-3767 to -3742 bp) as a probe and nuclear extracts prepared from chick embryo hepatocytes. Nuclear

extracts were incubated with the region E probe in the presence of different concentrations of unlabeled competitor DNAs. Each reaction contained 10 μg of nuclear protein. DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels. (A) Possible binding configurations of Pbx-Meis1 on region E. The position of putative tetrameric half-sites in region E are indicated by the boxes above the wild-type sequence for region E. The half-sites are labeled 1-4. The sequence of oligonucleotide competitors containing mutations of one or more half-sites is also shown. mut 1 to mut 4 refer to the number of the half-site that was mutated in each oligonucleotide. (B) Competition analysis with native and mutant forms of region E. Unlabeled competitor DNAs (2-, 5-, 20-, or 50-fold molar excess) were mixed with the radiolabeled probe prior to the addition of nuclear extract. These data are representative of four experiments employing independent preparations of nuclear extract.

Each of the Pbx1-Meis1 binding configurations on region E overlaps with each other suggesting that complex a and complex b are dimeric rather than tetrameric complexes. To confirm this supposition, gel mobility shift experiments were performed with region E probes that contained mutations in half-sites 1 and 2 (mut E 1/2) or half-sites 2 and 3 (mut E 2/3). Both of these probes bound hepatic nuclear proteins and *in vitro* synthesized Pbx1a, Pbx1b, and Meis1 in a pattern identical to that of the wild-type region E probe (data not shown). Thus, protein binding to region E represents dimers of Pbx1a, Pbx1b, and Meis1 and not higher order structures of these proteins.

Next, we investigated the effects of site-specific mutations of region E on T3 regulation of promoter activity in chick embryo hepatocytes. Hepatocytes were transiently transfected with reporter constructs containing mutations of two of the four half-sites of region E in the context of p[ME-3921/-3631]ME-147/+31CAT. A block mutation of region E extending from -3761 to -3744 (mut E block, Fig. 7A) inhibited T3 responsiveness by 58% (Fig. 8). This effect was due to a decrease in promoter activity in the presence of T3. Site-specific mutations that abolished protein binding to region E (i.e. mut E 1/3, mut E 1/4, and mut E 2/4) also inhibited T3 responsiveness and the extent of this inhibition was similar to that of the block mutation of region E. Site-specific mutations that partially inhibited (i.e. mut E 3/4) or had no effect (i.e. mut E 1/2 and mut E 2/3) on protein binding to region E caused a smaller decrease in T3 responsiveness compared to mut E 1/3, mut E 1/4, and mut E 2/4. Mut E 2/3 caused the smallest decrease in T3 responsiveness of all of the mutations examined. These data support the *in vitro* DNA binding results demonstrating that Pbx-Meis1 heterodimers bind region E in three configurations. Of these configurations, binding to half-sites 1 and 4 confers the highest level of transcriptional activation. Interestingly, this site perfectly matches the consensus binding sequence for Pbx1-Meis1 heterodimers (i.e. TGATTGAC) except that the tetrameric half-sites are separated by a 7 bp spacer. This is the first description of a native Pbx1-Meis1 binding sequence with this arrangement of half-sites.

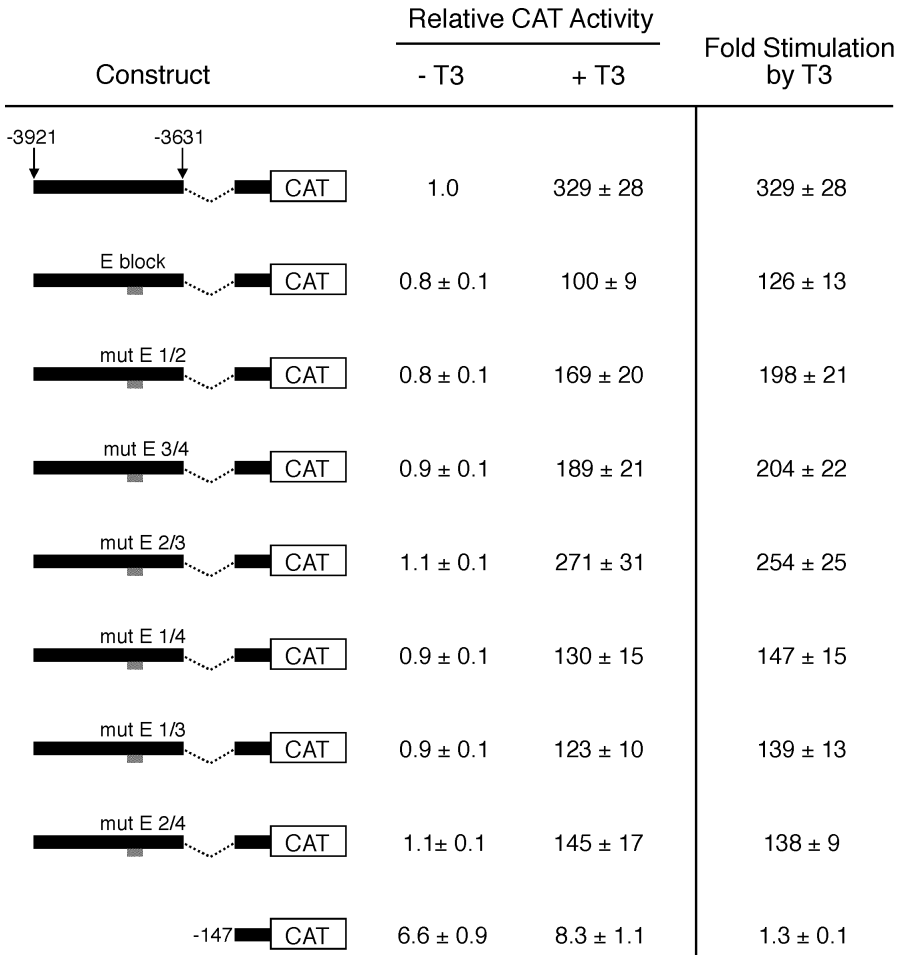

Figure 8. Stimulation of T3 responsiveness by region E is correlated with the binding activity of complex a and complex b.

p[ME-3921/-3631]ME-147/+31CAT or constructs containing mutations of region E in the context of p[ME-3921/-3631]ME-147/+31CAT were transiently transfected into chick embryo hepatocytes as described under "Experimental Procedures". Transfections also contained CMV-β-galactosidase as a control for transfection efficiency. After the transfection, cells were treated with or without T3 for 48 h. Cells were then harvested, extracts prepared, and CAT and β-galactosidase assays performed. (Left) the constructs used in these experiments. Numbers indicate the 5' or 3' boundaries of malic enzyme DNA in nucleotides relative to the start site for transcription. The mutated sequences in region E are shown in Fig. 7A. (Right) CAT activity was expressed relative to β-galactosidase activity to correct for differences in transfection efficiency. CAT activity in cells transfected with p[ME-3921/-3631]ME-147/+31CAT and incubated without T3 was set at 1, and the other activities were adjusted proportionately. The -fold stimulation by T3 was calculated by dividing CAT activity in hepatocytes treated with T3 (+T3) by that for hepatocytes not treated with T3 (-T3). The -fold responses were calculated for individual experiments and then averaged. The results are the means ± S.E. of 5 experiments.

Region E can function in isolation to enhance T3 regulation of transcription.

Pbx-Meis1 bound to region E (-3762/-3748 bp) may enhance T3 regulation of malic enzyme transcription by interacting with TR-RXR heterodimers bound to the T3RU (-3883/-3769 bp). Alternatively, Pbx-Meis1 may enhance T3 responsiveness by interacting with other TR accessory factors bound to sequences flanking the T3RU. For example, Pbx-Meis1 may interact with CCAAT/enhancer-binding protein-α bound to region F (-3703/-3686 bp). To assess the role of these possible mechanisms of Pbx-Meis1 action, transient transfection experiments were conducted to determine whether region E could function in isolation to enhance T3 regulation of transcription conferred by a T3RE. Within the malic enzyme T3RU is a strongly active T3RE designated as T3RE2 (-3883/-3858 bp). When hepatocytes were transfected with a reporter plasmid containing T3RE2 linked to the minimal promoter of the malic enzyme gene (p[T3RE2]ME-147/+31CAT), T3 stimulated a 10-fold increase in promoter activity (Fig. 9). When a DNA fragment containing region E (-3767/-3742 bp) was inserted downstream of T3RE2 in p[T3RE2]ME-147/+31CAT, the stimulation of promoter activity caused by T3 was increased to more than 46-fold. The increase in T3 responsiveness caused by region E was due to an increase in promoter activity in the presence of T3. Insertion of DNA fragment containing mut E block downstream of T3RE2 in p[T3RE2]ME-147/+31CAT had no effect on T3 responsiveness, indicating that the increase in T3 responsiveness caused by region E was not due to changes in the spacing between T3RE2 and the minimal malic enzyme promoter. Region E also enhanced T3 responsiveness when it was ligated downstream to the malic enzyme T3RU in p[ME-3921/-3766]ME-147/+31CAT (data not shown). These data indicate that the stimulation of T3-induced malic enzyme transcription caused by region E is mediated by interactions between Pbx-Meis and TR-RXR.

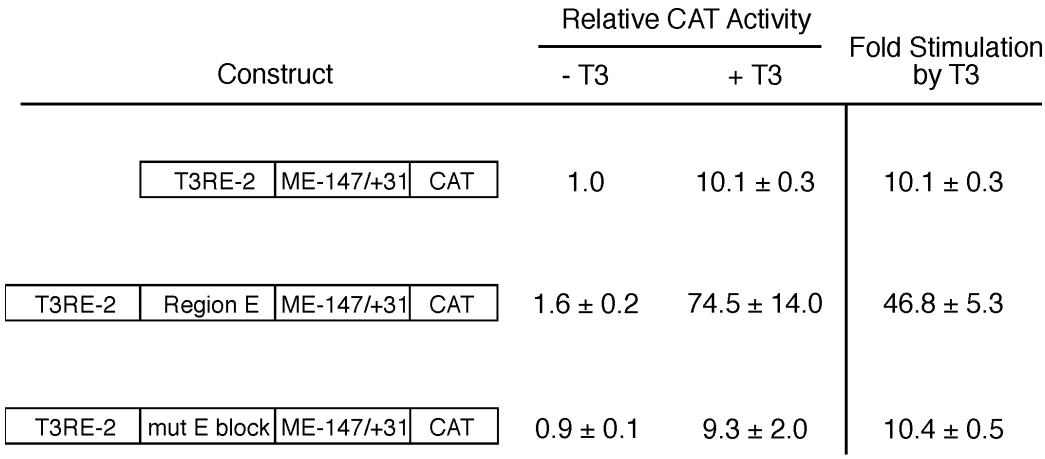

Figure 9. Region E can function in isolation to enhance T3 regulation of transcription.

Oligonucleotides containing region E (-3767 to -3742 bp) or a block mutation of region E (mut E block) were inserted downstream of malic enzyme T3RE2 in p[T3RE2]ME-147/+31CAT. Hepatocytes were transiently transfected with these constructs and treated with or without T3 as described in the legend to Fig. 8. and under "Experimental Procedures". (Left) constructs used in these experiments. The sequence for region E and mut E block are shown in Fig. 7A. (Right) CAT activity in cells transfected with p[T3RE2]ME-147/+31CAT and treated without T3 was set at 1, and the other activities were adjusted proportionately. The -fold stimulation by T3 was calculated as described in the legend in Fig. 8. The results are the means ± S.E. of 5 experiments.

Pbx1 physically interacts with TR.

Previous studies have demonstrated that Pbx-Meis1 heterodimers function as cofactors for other transcription factors (Knoepfler et al., 1999). For example, Pbx1-Meis1 bound to a PCE strongly augments transcriptional activation by adjacent myogenic regulatory complexes containing MyoD or myogenin. This effect of Pbx1-Meis1 is mediated, in part, by direct interactions between the Pbx1 homeodomain and a highly conserved tryptophan motif flanking the basic helix-loop-helix domain of MyoD and myogenin. These findings coupled with the close proximity of region E to the T3RU prompted us to hypothesize that Pbx1-Meis1 enhanced T3 responsiveness of malic enzyme transcription by directly interacting with TR-RXR. To investigate this hypothesis, we determined whether Pbx1 or Meis1 physically interacted with TR and RXR using a pull-down assay. In our initial experiments, we investigated the ability of *in vitro* synthesized TRα and RXRα to bind a bacterially expressed fusion protein containing glutathione S-transferase (GST) linked to Pbx1a. ^{35}S-labeled TRα bound to GST-Pbx1a in the absence and presence of unlabeled RXRα, and the interaction between ^{35}S-labeled TRα and GST-Pbx1a was enhanced by more than two-fold by the presence of T3 (Fig. 10B). ^{35}S-labeled TRα also interacted with GST-Pbx1b in a T3 regulated manner (Fig. 10C). No interaction was observed between ^{35}S-labeled TRα and GST. In contrast to the results for TRα, little or no interaction was observed between ^{35}S-labeled RXRα and GST-Pbx1a (Fig. 10B) and between ^{35}S-labeled RXRα and GST-Pbx1b (Fig. 10C). The lack of interaction between RXRα and Pbx1 proteins was confirmed by pull down experiments employing GST-RXRα as the bait and ^{35}S-labeled Pbx1a and Pbx1b synthesized *in vitro* (data not shown). These data indicate that Pbx1a and Pbx1b selectively interact with TR of the TR-RXR heterodimeric complex.

Pull-down assays were also carried out using GST-TRα as the bait protein. Consistent with results of experiments employing GST-Pbx1a and GST-Pbx1b as bait proteins, ^{35}S-labeled Pbx1a and Pbx1b strongly interacted with GST-TRα (Fig. 11). In contrast, a very weak interaction was observed between ^{35}S-labeled Meis1 and GST-TRα in the absence or presence of unlabeled Pbx1a. These data further demonstrate that interactions between Pbx1 proteins and TRα are specific. In contrast to results of experiments analyzing interactions between GST-Pbx1 proteins and ^{35}S-labeled TRα, interactions between GST-TRα and ^{35}S-labeled Pbx1 proteins

were not affected by the presence of T3. The latter observation suggests that appending GST to the N-terminus of TRα blocks ligand-induced conformational changes that facilitate interactions between TRα and Pbx1 proteins. One possible mechanism is that addition of GST to the N-terminus of TRα causes the conformational change of TRα. As a result, T3 can no longer make certain residues in TRα exposed to enhance the interaction between Pbx1 and TRα.

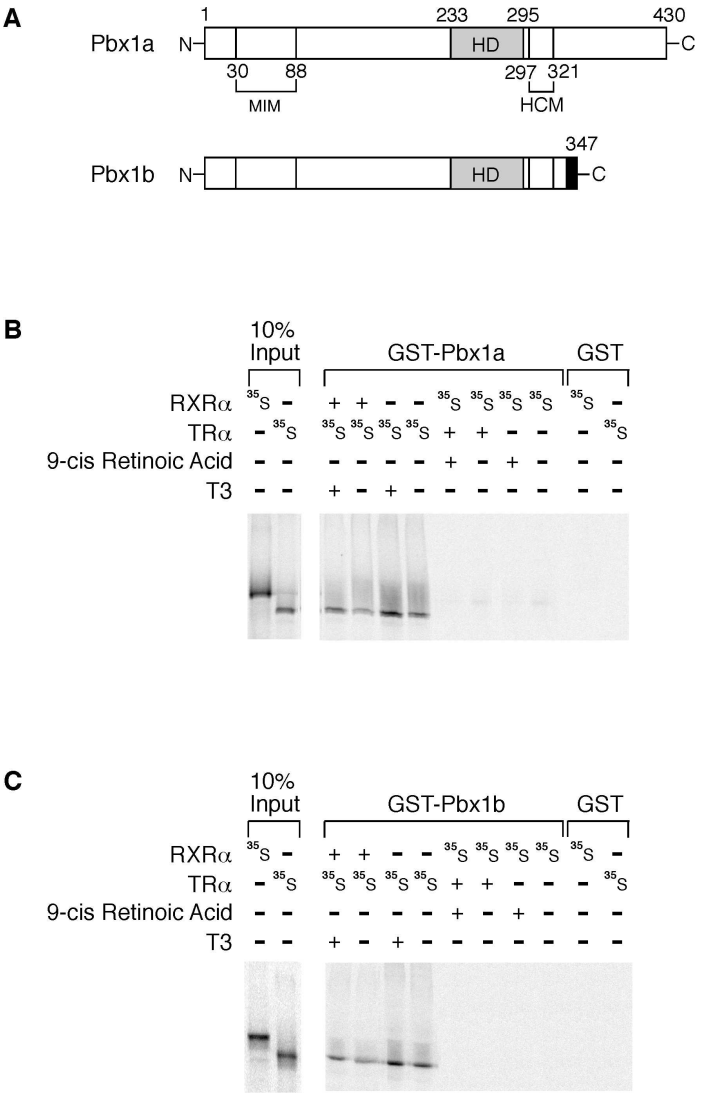

Figure 10. TRα interacts with Pbx1a and Pbx1b in a T3 regulated manner.

(A) Schematic representation of Pbx1a and Pbx1b. The Meis1 interaction motif (MIM), homeodomain (HD) region, and Hox cooperativity motif (HCM) are indicated. (B) TRα interacts with GST-Pbx1a. Bacterially produced GST or GST-Pbx1a immobilized on glutathione-sepharose beads was incubated with *in vitro* translated and ^{35}S-labeled TRα or RXRα as described in "Experimental Procedures". Some incubations with ^{35}S-labeled TRα contained an equimolar amount of unlabeled RXRα, and some incubations with ^{35}S-labeled RXRα contained an equimolar amount of unlabeled TRα. Incubations were performed in the absence and presence of 1 μM T3 or 1 μM 9-cis retinoic acid as indicated. After the matrix was extensively washed, labeled proteins retained on the beads were eluted, resolved by SDS-PAGE, and visualized by storage phosphor autoradiography together with 10% of the total radiolabeled receptor input used in each binding reaction. (C) Incubations were performed as described in panel B except that GST-Pbx1b was used as the bait protein. These data are representative of four experiments.

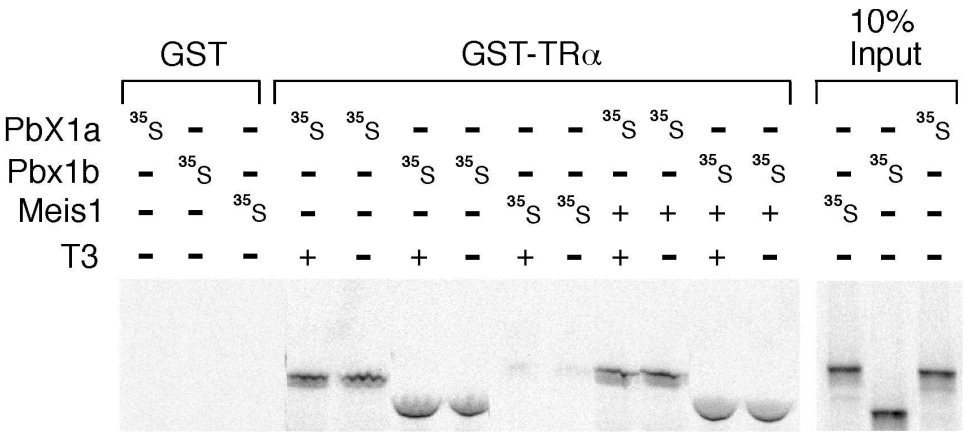

Figure 11. TRα strongly interacts with Pbx1a and Pbx1b but not Meis1.

GST or GST-TRα immobilized on glutathione-sepharose beads was incubated with *in vitro* translated and ^{35}S-labeled Pbx1a, Pbx1b, or Meis1 in the absence and presence of T3. An equimolar amount of unlabeled Meis1 was included in some incubations containing ^{35}S-labeled Pbx1a and Pbx1b. After the matrix was extensively washed, labeled proteins retained on the beads were eluted, resolved by SDS-PAGE, and visualized by storage phosphor autoradiography together with 10% of the total radiolabeled protein input used in each binding reaction. Additional experimental details are described in "Experimental Procedures".

To map the motifs in TRα that interact with Pbx1a, pull-down experiments were conducted using various truncations of TRα labeled with ^{35}S *in vitro*. Deletion of the first 20 amino acids from the N-terminus of TRα had little or no effect on the binding of TRα to GST-Pbx1a (Fig. 12). Further deletion to amino acid 51 decreased the binding of TRα to GST-Pbx1a. When deletion of the N-terminus of TRα was extended to amino acid 120, binding of TRα to GST-Pbx1a was abolished. To further analyze the interaction between TRα and Pbx1a, TRα polypeptides containing amino acids 1 to 157, amino acids 1 to 118, and amino acids 51 to 157 were tested for their ability to interact with GST-Pbx1a. A strong interaction was observed between GST-Pbx1a and a TRα polypeptide containing amino acids 1 to 157. Markedly weaker but detectable interactions were observed between GST-Pbx1a and TRα polypeptides containing amino acids 1 to 118 and amino acids 51 to 157. GST-Pbx1b bound to TRα truncations in a manner similar to that of GST-Pbx1a (data not shown). Collectively, these data suggest that the N-terminal region of TRα containing the DNA binding domain plus additional flanking sequences (amino acids 21 to 157) is required for optimal binding to Pbx1a and Pbx1b. Interestingly, interactions between GST-Pbx1 fusion proteins and TRα truncations lacking the ligand binding domain were not enhanced by the presence of T3. This observation is consistent with the scenario that T3 binding to the ligand binding domain of TRα causes a conformational change that enhances the ability of the N-terminal region of TRα to interact with Pbx1a and Pbx1b.

We next investigated the ability of N-terminal truncations of Pbx1a to interact with GST-TRα. Deletion of the first 79 amino acids of Pbx1a containing the Meis1 interaction motif had no effect on the binding of Pbx1a to GST-TRα (Fig. 13). Further deletion to amino acid 119 resulted in a 30% increase in binding of Pbx1a to GST-TRα. These data indicate that TRα interacts with a Pbx1a region (amino acids 119 to 430) containing the homeodomain plus additional flanking amino acids. In addition, Pbx1a contains a motif between amino acids 80 to 118 that inhibits the interaction between Pbx1a and TRα.

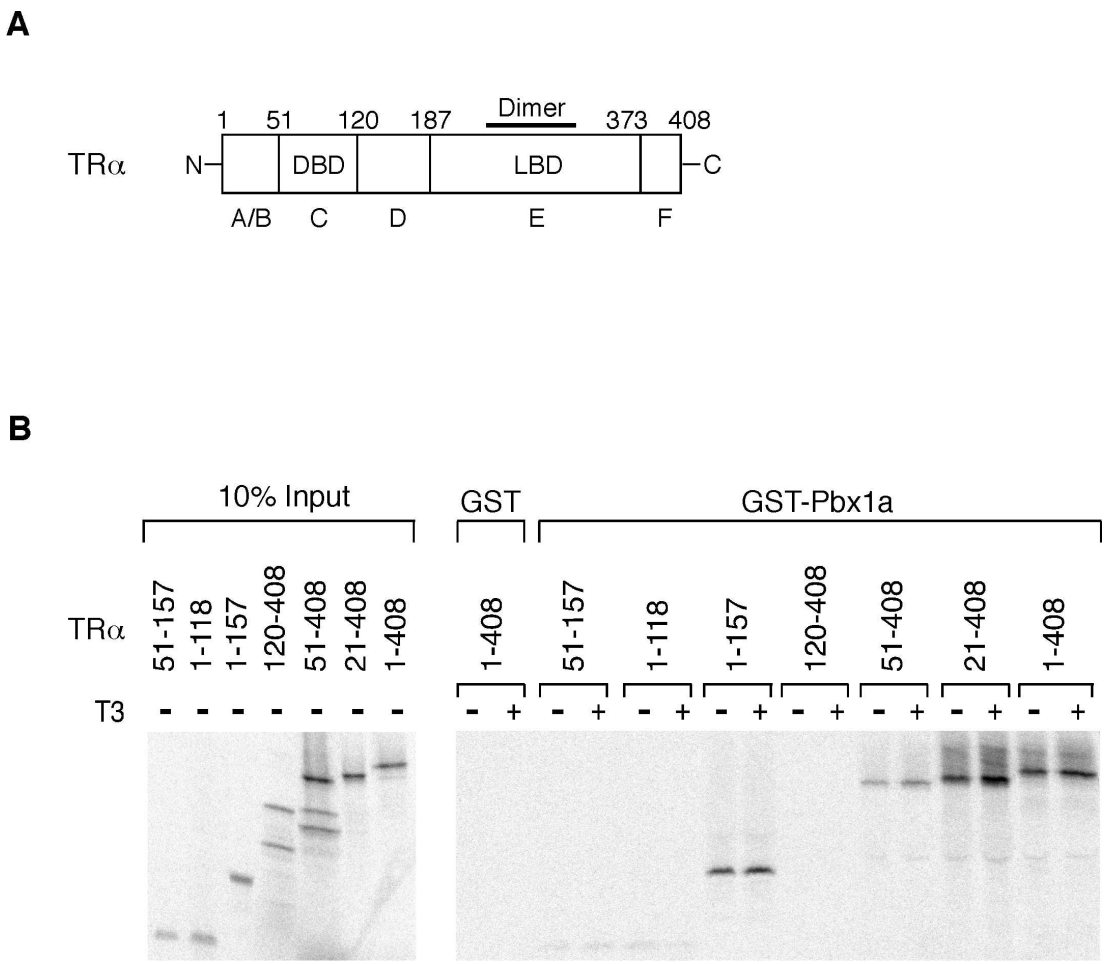

Figure 12. A region of TRα containing amino acids 21 to 157 is required for optimal binding to Pbx1a.

The schematic representation of TRα. The DNA binding domain (DBD) and the ligand binding domain (LBD) are indicated. Embedded within the LBD of TRα is a heptad repeat region involved in dimerization. A ligand-induced change in the conformation of the LBD is responsible for providing an interface for interaction with coactivator proteins. (B) Wild-type and truncated forms of TRα labeled with ^{35}S by *in vitro* translation were incubated with GST or GST-Pbx1a bound to glutathione-sepharose beads. After the matrix was extensively washed, labeled proteins retained on the beads were eluted, resolved by SDS-PAGE, and visualized by storage phosphor autoradiography together with 10% of the total radiolabeled protein input used in each binding reaction.

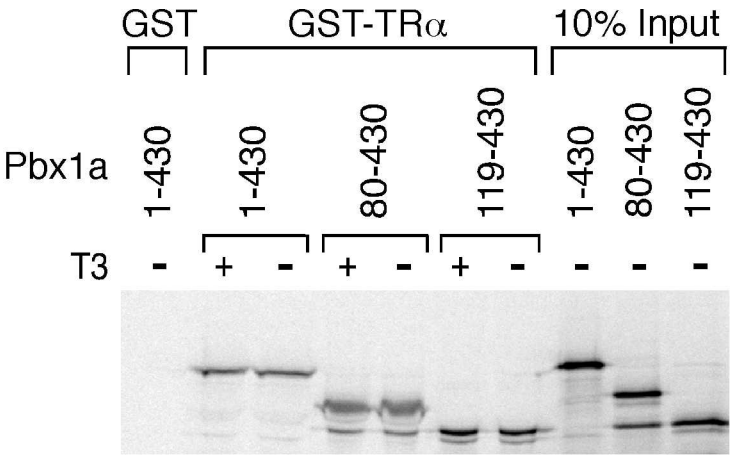

Figure 13. TRα interacts with a region of Pbx1 that is distinct from the Meis1 interaction motif.

Wild type (amino acids 1-430) and truncated forms of Pbx1a lacking the Meis1 interaction domain (amino acids 80-430 and amino acids 119-430) were labeled with ^{35}S by *in vitro* translation. These proteins were incubated with GST or GST-TRα bound to glutathione-sepharose beads. After washing, labeled proteins retained on the beads were eluted, resolved by SDS-PAGE, and visualized by storage phosphor autoradiography together with 10% of the total radiolabeled protein input used in each binding reaction.

D. Discussion

Protein complexes containing Pbx and Meis1/Prep1 play a critical role in embryonic development in both invertebrates and vertebrates (Lumsden and Krumlauf, 1996; Burglin, 1997; Popperl et al, 2000). While less is known about the functions of Pbx-Meis1/Prep1 in adult tissues, data from recent studies suggest that these complexes are involved in controlling endocrine function. For example, Pbx-Meis1 complexes mediate the stimulatory effects of cAMP on transcription of 17α-hydroxylase cytochrome P450, a steroid hydroxylase involved in the production of steroid hormones from cholesterol (Bischof et al., 1998;). Other studies have shown that Pbx-Prep1 complexes are involved in mediating the pancreatic-specific expression of the genes for somatostatin and glucagon (Goudet et al., 1999; Herzig et al., 2000). Still other studies have demonstrated that interactions between Pbx complexes and the glucocorticoid receptor mediate the inhibitory effect of glucocorticoids on prolactin transcription in non-pituitary cell lines (Subramaniam et al., 1998). In the present study, we show that Pbx-Meis1 complexes interact with TR-RXR complexes to enhance T3 regulation of malic enzyme transcription in hepatocytes. These studies establish a role for Pbx-Meis1 in the regulation of thyroid hormone action.

Data from transfection (Fig. 8 and Fig. 9) and protein binding assays (Fig. 10 and Fig. 11) suggest that stimulation of T3-induced malic enzyme transcription by region E is mediated by physical interactions between Pbx-Meis1 and TR-RXR. Due to the close proximity of region E to the T3RU, Pbx-Meis1 may bind the malic enzyme gene as a tetrameric complex with TR-RXR. In support of this possibility, peptide sequences mediating the interaction between Pbx1 and TRα are distinct from those mediating interactions between Pbx1a/b and Meis1 and between TRα and RXRα (Fig. 12 and Fig. 13). Furthermore, in gel mobility shift assays, *in vitro* synthesized TRα, RXRα, Pbx1a/b, and Meis1 form a complex containing all four proteins on an oligonucleotide probe consisting of T3RE2 linked to region E (data not shown). Because tetrameric complex formation does not alter the total binding of TRα-RXRα and Pbx1a/b-Meis1 to the probe, the mechanism by which region E modulates T3 responsiveness does not appear to involve changes in DNA binding. Previous studies have shown that the stimulatory effects of T3

on transcription are mediated by the recruitment of coactivator proteins to the TR (Onate et al., 1995). Coactivators of TR may regulate transcription by directly interacting with the basal transcriptional machinery, by modulating interactions between TR and the basal transcriptional machinery, and by modifying chromatin structure. We hypothesize that complex formation between TR-RXR and Pbx-Meis1 augments T3-induced malic enzyme transcription by facilitating the recruitment of coactivators to the malic enzyme promoter.

A role for accessory DNA binding factors in modulating T3 responsiveness has been described for other genes. For example, the T3-induced stimulation of the phosphoenolpyruvate carboxykinase (PEPCK) promoter in hepatoma cells is mediated by a T3RE and a sequence that binds the liver-enriched factors, C/EBPα and C/EBPβ (Park et al., 1990). In primary hepatocyte cultures, T3 stimulation of the S14 gene requires the presence of T3REs located between -2700 to -2500 bp and a nuclear factor-Y (NF-Y) binding site near the transcription start site (Jump et al., 1997). Increased T3 responsiveness of the rat growth hormone promoter in pituitary cells relative to non-pituitary cells is mediated by a synergistic interaction between TR and the pituitary-specific DNA binding protein, Pit-1 (also referred to as GHF-1). Physical interactions between Pit-1 and TR enhance the binding of TR to the rat growth hormone promoter (Palomino et al., 1998). In cardiac muscle cells, myocyte-specific enhancer factor 2 (MEF2) potentiates the ability of TR to stimulate α-cardiac myosin heavy-chain transcription in the presence of T3 (Lee et al., 1997). Protein binding assays indicate that MEF2 and TR specifically bind each other. Collectively, these findings and the results of the present study indicate that a wide variety of transcription factors can interact with TR on promoters of T3 responsive genes. Such factors expand the range of hormone responsiveness and constitute the primary mechanism by which T3 regulates transcription in a tissue- or cell-type-specific manner.

Complexes containing Pbx-Meis1/Prep1 have been shown to function as accessory proteins for other transcription factors besides TR. For example, in the urokinase plasminogen activator (uPA) promoter, Pbx-Prep1 binds a cooperation mediating (COM) element that is positioned between an AP-1 site and a combined PEA3/AP-1 site (Berthelsen et al., 1998). Binding of Pbx-Prep1 to the COM element is required for synergistic activation of uPA transcription by the AP-1 and PEA3/AP-1 sites. The elastase I promoter contains a Pbx binding site that comprises part of an enhancer element conferring cell-type-specific regulation of transcription (Kruse et al., 1995). In pancreatic acinar cells, this Pbx binding site potentiates the

transcriptional activity of an adjacent element that binds the pancreatic acinar cell-specific factor, PTF-1 (Swift et al., 1998). The somatostatin promoter contains adjacent binding sites for Pbx-Prep1 and the pancreatic cell-specific factor, PDX1 (Peers et al., 1995). Pbx-Prep1 alone is devoid of transcriptional activity but interacts with PDX1 to stimulate a 10-fold increase in somatostatin promoter activity. This functional interaction between Pbx-Prep1 and PDX1 is not mediated by alterations in the binding of Pbx-Prep1 and PDX1 to the somatostatin promoter. E2a-MyoD heterodimers in muscle cell nuclear extracts bind to E-box motifs that are flanked at their 5' end by a PCE (Knoepfler et al., 1999). In transfection assays, the binding of Pbx-Meis1 to the PCE enhances transcriptional activation by E2a-MyoD bound to the adjacent E-box motif. In contrast to the interaction between Pbx-Prep1 and PDX1 on the somatostatin promoter and the interaction between Pbx-Meis1 and TR-RXR on the malic enzyme promoter, the interaction between Pbx-Meis1 and E2a-MyoD on the PCE/E-box element is mediated, in part, by alterations in protein binding to DNA. These studies indicate that Pbx-Meis1/Prep1 complexes functionally interact with a wide variety of transcription factors and that the mechanisms mediating these interactions vary depending on the nature of the proteins that interact with Pbx-Meis1/Prep1.

Several coactivators of TR have been identified that enhance T3-dependent transcriptional activation by physically interacting with the ligand binding domain (LBD) of TR (Onate et al., 1995; Ikeda et al., 1999). Examples of such proteins include CREB binding protein (CBP) and steroid receptor coactivator 1 (SRC1). T3-dependent transcriptional activation is also controlled by nuclear proteins that physically interact with TR regions distinct from the LBD. For example, the general transcription factor, TFIIB, facilitates T3-dependent transcriptional activation by interacting with a ten amino acid sequence in the A/B region of TRα (Tong et al., 1995). MEF2 and the hematopoietic bZIP protein p45/NF-E2 enhance T3-dependent transcriptional activation by interacting with the DNA binding domain of TR (Lee et al., 1997; Cheng et al., 1997). Results from the present study demonstrating that Pbx1a/b interacts with the N-terminal region of TRα provide further support for a role of TR regions distinct from the LBD in modulating T3-dependent transcriptional activation.

Results from DNA binding analyses (Fig. 7) indicate that region E contains a novel PCE that binds Pbx-Meis1 heterodimeric complexes in one of three possible configurations. One configuration is comprised of tandem half-sites separated by a 7 bp spacer (i.e. half-sites 1 and 4)

and the other two configurations are comprised of tandem half-sites with no spacer sequence (i.e. half-sites 1 and 2 and half-sites 3 and 4). Results from transfection experiments suggest that all three binding configurations contribute to the stimulation of T3 responsiveness by region E with binding to half-sites 1 and 4 making the greatest contribution. Interestingly, site-specific mutations of region E that restricted Pbx-Meis1 binding to half-sites 1 and 4 (mut E 2/3) or half-sites 3 and 4 (mut E 1/2) had no effect on the DNA binding affinity yet conferred a smaller increase in T3 responsiveness than wild-type region E. This observation suggests that the distance between or sequences flanking the half-sites of these Pbx-Meis1 binding configurations can modulate the functional activity of Pbx-Meis1 complexes without altering their ability to bind DNA.

We previously reported that region E was one of four cis-acting elements mediating differences in T3 regulation of malic enzyme transcription between chick embryo hepatocytes and chick embryo fibroblasts (Fang and Hillgartner, 1998). This conclusion was based on the finding that mutation of region E markedly inhibited T3 regulation of malic enzyme transcription in chick embryo hepatocytes but had no effect on T3 regulation in chick embryo fibroblasts. Cell-type dependent differences in region E activity are probably not due to alterations in expression of Pbx and Meis1, as these proteins are expressed in a wide variety of tissues. This supposition is supported by data from gel mobility shift assays demonstrating that the pattern and extent of protein binding to region E in chick embryo fibroblasts is similar to that observed in chick embryo hepatocytes (Fang and Hillgartner, 1998). We postulate that the difference in region E activity between chick embryo hepatocytes and chick embryo fibroblasts is mediated by a post-translational mechanism. One possibility is that Pbx-Meis1 interacts with an activator or inhibitor whose activity varies in different cell types. In support of this possibility, Abramovich et al. (2000) have recently identified a novel Pbx-interacting protein that inhibits the ability of Pbx complexes to bind DNA and activate transcription. Further studies are needed to determine the role of this protein in mediating differences in T3 responsiveness between chick embryo hepatocytes and chick embryo fibroblasts.

In summary, these studies provide novel data demonstrating a role for Pbx and Meis1 in the control of gene transcription in hepatocytes. Pbx1-Meis complexes may regulate the transcription of other genes in liver besides malic enzyme. For example, the AC element of the PEPCK promoter contains a sequence (-255 to -248 bp) that perfectly matches the consensus

PCE, TGATTGAC. Mutation of the AC element abrogates the effects of cAMP and insulin on PEPCK transcription in H4IIe hepatoma cells (Yeagley et al., 2000). Additional experimentation will be needed to determine whether Pbx-Meis1 complexes play a role in the regulation of transcription of other metabolically important genes in liver.

Chapter 3. CCAAT/Enhancer-Binding Protein (C/EBP) Enhances T3 Responsiveness of Malic Enzyme Gene in Chick Embryo Hepatocytes

A. Introduction

C/EBP is a member of the basic region-leucine zipper (bZIP) class of transcription factors. C/EBP family consists of at least six members, including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and CHOP which bind to specific DNA sequences as homo- or heterodimers (Yano et al., 1996). C/EBPα was the first member in this family to be purified and cloned (Johnson et al., 1987). C/EBPα is abundant in liver and adipose tissues and will bind to and activate transcription of several liver- and adipose-specific promoters including aP2-422 (Cheneval et al., 1991), phosphoenolpyruvate carboxykinase (PEPCK) (Park et al., 1990), glucose transporter 4 (GLUT4) (Kaestner et al., 1990) and C/EBPα itself (Christy et al., 1991).

Previous work has demonstrated that C/EBP plays an important role in development and maintenance of metabolically important processes. One extensively studied gene regulated by C/EBP is phosphoenolpyruvate carboxykinase (PEPCK). C/EBPα and C/EBPβ have been implicated in the control of PEPCK gene transcription (Park et al., 1996; Park et al., 1993). They bind to the PEPCK gene promoter and can stimulate transcription from the promoter when transfected into hepatoma cells. C/EBPα knockout mice exhibit profound derangement of liver structure and function, such as disturbed hepatic architecture with acinar formation (Flodby et al., 1996; Wang et al., 1995). In addition, c-Myc and c-Jun expression is induced in liver, consistent with a stimulation of cell proliferation. Brown adipose tissue and white adipose tissue also fail to develop normally in C/EBPα knockout mice. Because C/EBPα knockout mice exhibit low expression of PEPCK and glucose 6-phosphatase, they can not synthesize glucose to maintain glucose homeostasis in the perinatal period.

Previous work in our lab has demonstrated that inserting a block mutation into region F causes a 55% decrease in T3 responsiveness of the malic enzyme promoter in CEH (Fang and Hillgartner, 1998). Data from *in vitro* DNA-binding assays indicate that C/EBPα binds to region

F. To obtain further evidence indicating the involvement of C/EBPα in controlling the T3 responsiveness of the malic enzyme promoter, C/EBPα activity in CEH was inhibited by transfecting an expression plasmid that expresses a dominant negative protein targeted against C/EBPα. In addition, *in vitro* protein interaction assays were performed to determine whether C/EBPα interacts with TR and RXR.

B. Experimental Procedures

Plasmids - The DNA fragments used to construct reporter plasmids were named by designating the 5' and 3' ends of each fragment relative to the start site of transcription of the malic enzyme gene. p[T3RE2]ME-147/+31CAT was made by subcloning the major malic enzyme T3RE between -3883 to -3858 bp into the BamH I/Sac I site upstream of the minimal promoter in ME-147/+31CAT. p[T3RE2+F]ME-147/+31CAT was constructed by inserting region F (ME-3703/-3686) into the Spe I/Sma I site immediately downstream of the T3RE2 in p[T3RE2]ME-147/+31CAT. Dr. C. Vinson (National Cancer Institute) provided us with an expression vector that expresses a C/EBPα-specific, dominant negative protein, which is termed A-C/EBP, from the Rous sarcoma virus (RSV) promoter. Human RXRα (RXRα 1-462) was subcloned into pSV-SPORT1 (GIBCO/BRL). The full-length cDNA of chicken TRα corresponding to amino acids 1 to 408 (TRα 1-408) was subcloned into pGEM-3Zf(-) (Promega). N-terminal and C-terminal deletion derivatives of chicken TRα were generated by PCR. PCR products encoding TRα polypeptides containing amino acids 1-118, 1-157, 21-408, 51-408, and 120-408 were subcloned into pGEM-3Zf(-). To generate plasmids that express fusion proteins containing glutathione S-transferase (GST) linked to C/EBPα and TRα, cDNA containing the full-length coding region of C/EBPα or TRα was subcloned into pGEX-2T (Amersham Pharmacia). Structures of reporter plasmids and expression plasmids were confirmed by restriction enzyme mapping and nucleotide sequence analyses.

Cell Culture and Transient Transfection - Primary cultures of chick embryo hepatocytes were prepared as previously described (Goodridge, 1973) and maintained in serum-free Waymouth

medium MD705/1 containing 50 nM insulin (gift from Eli Lilly Corp.) and corticosterone (1 μM). Hepatocytes were incubated at 40°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were transfected using a modification of the method of Baillie et al. (Baillie et al., 1993). Briefly, chick embryo hepatocytes were isolated as described above and plated on 35 mm dishes. At 18 h of incubation, the medium was replaced with one containing 20 μg LipofectACE (GIBCO/BRL), 3.0 μg of p[T3RE2+F]ME-147/+31CAT or an equimolar amount of another reporter plasmid, 0.05 μg pRSV-A-C/EBP or empty vector, and 0.05 μg of pCMV-β-galactosidase. At 42 h of incubation, the transfection medium was replaced with fresh medium with or without T3 (1.5 μM). At 90 h of incubation, CEH were harvested and cell extracts were prepared for CAT (Gorman et al., 1982) and β-galactosidase (Sambrook et al., 1989) measurements. CAT activity was expressed relative to β-galactosidase activity to correct for differences in transfection efficiency between samples. All DNAs used in transfection experiments were purified using the Qiagen endotoxin-free kit.

Protein-protein interactions - GST or GST fusion proteins were expressed in *E. coli.* (BL21, pLysS) and purified using standard techniques (Smith and Johnson, 1988). Briefly, bacteria were transformed with pGEX-2T or recombinant pGEX-2T plasmids expressing GST fusion proteins. Overnight bacterial cultures in ampicillin (250 μg/ml) were diluted 1:100 into 250 ml of Luria broth and grown at 37°C to an $O.D._{600} = 1.0$ before induction with 1 mM isopropylthiogalactopyranoside (IPTG) for 60 min. Cells were pelleted and resuspended in 5 ml of buffer A (50 mM KCl, 25 mM HEPES [pH 7.9], 6% glycerol, 5 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiotheritol, 0.05% Triton X-100). Cells were lysed on ice by sonication and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was mixed for 1 h at 4°C on a rotator with 0.5 to 1 ml of 50% glutathione-sepharose beads (Amersham Pharmacia) which were preswollen in buffer A. After absorption, beads were collected by centrifugation at 4°C and washed three times with 1 ml of buffer A. Fusion proteins coupled to the glutathione-sepharose beads were stored at 4°C as 50% (v/v) slurry in buffer A. The concentrations and sizes of GST and GST fusion proteins were estimated by SDS-PAGE, using a known quantity of molecular weight standards.

L-[^{35}S]cysteine- or L-[^{35}S]methionine-labeled proteins were prepared by using TNT reticulocyte lysates (Promega). Approximately 2.5 x 10^4 to 5 x 10^4 cpm of ^{35}S-labeled protein was incubated with 100 ng of GST fusion protein immobilized on glutathione-sepharose beads in

300 µl of buffer A for 1 h at 4°C on a rotator. Beads were collected by centrifugation at 4°C and washed three times with 1 ml of buffer A. The bound proteins were eluted with SDS-gel loading buffer and analyzed by SDS-PAGE followed by storage phosphor autoradiography. Where indicated, 1 µM T3 was included in the binding reaction mixture. ^{35}S-labeled proteins were analyzed by electrophoresis and storage phosphor autoradiography to ensure that equal amounts of input radioactivity of the labeled protein were used in the GST binding assays.

C. Results

Region F can function in isolation to enhance T3 regulation of transcription.

C/EBP bound to region F (-3703 and -3686 bp) may enhance T3 regulation of malic enzyme transcription by interacting with TR-RXR heterodimers bound to the T3RU (-3883 and -3769 bp). Alternatively, C/EBP may enhance T3 responsiveness by interacting with other TR accessory factors bound to sequences flanking the T3RU. To assess the role of these possible mechanisms of C/EBP action, transient transfection experiments were conducted to determine whether region F could function in isolation to enhance T3 regulation of transcription conferred by a T3RE. Within the malic enzyme T3RU is a strongly active T3RE designated as T3RE2 (-3883/-3858 bp). When hepatocytes were transfected with a reporter plasmid containing T3RE2 linked to the minimal promoter of the malic enzyme gene (p[T3RE2]ME-147/+31CAT), T3 stimulated a 10-fold increase in promoter activity (Fig. 14). When a DNA fragment containing region F (-3703/-3686 bp) was inserted downstream of T3RE2 in p[T3RE2]ME-147/+31CAT, the stimulation of promoter activity caused by T3 was increased to 77-fold. The increase in T3 responsiveness caused by region F was due to an increase in promoter activity in the presence of T3. Insertion of DNA fragment containing mut F block downstream of T3RE2 in p[T3RE2]ME-147/+31CAT had no effect on T3 responsiveness, indicating that the increase in T3 responsiveness caused by region F was not due to changes in the spacing between T3RE2 and the minimal malic enzyme promoter. These data indicate that the stimulation of T3-induced malic enzyme transcription caused by region F may be mediated by interactions between C/EBP and TR-RXR.

Dominant negative C/EBP inhibits the T3-induced promoter activity in region F.

To provide functional evidence supporting the hypothesis that region F interacts with C/EBPα, a dominant negative form of C/EBPα termed A-C/EBP were cotransfected into CEH with the reporter plasmids used in Fig. 14. Expression of A-C/EBP inhibited p[T3RE2+F]ME-147/+31CAT activity by 80% (Fig. 15) relative to cells transfected with empty RSV vector. In contrast, expression of A-C/EBP had no effect on the T3 responsiveness of p[T3RE-2+mut F block]ME –147/+31CAT. These observations further support the hypothesis that C/EBP binds region F and enhances T3-induced transcription.

Construct				Relative CAT Activity - T3	Relative CAT Activity + T3	Fold Stimulation by T3
	T3RE-2	ME-147/+31	CAT	1.0	10.1 ± 0.3	10.1 ± 0.3
T3RE-2	Region F	ME-147/+31	CAT	0.9 ± 0.1	70.1 ± 17.9	77.1 ± 5.9
T3RE-2	mut F block	ME-147/+31	CAT	0.9 ± 0.1	9.3 ± 2.1	10.4 ± 0.5

Figure 14. Region F can function in isolation to enhance T3 regulation of transcription.

Oligonucleotides containing region F (-3702/-3686 bp) or a block mutation of region F (mut F block) were inserted downstream of malic enzyme T3RE2 in p[T3RE2]ME-147/+31CAT. Hepatocytes were transiently transfected with these constructs and treated with or without T3 as described in the legend to Fig. 8. and under "Experimental Procedures". (Left) constructs used in these experiments. (Right) CAT activity in cells transfected with p[T3RE2]ME-147/+31CAT and treated without T3 was set at 1, and the other activities were adjusted proportionately. The -fold stimulation by T3 was calculated as described in the legend in Fig. 8. The results are the means ± S.E. of 5 experiments.

Construct	Relative CAT Activity				Fold Stimulation by T3
	RSV vector		A-C/EBP		
	- T3	+ T3	- T3	+ T3	
T3RE-2 ME-147/+31 CAT	1.0	7.3 ± 0.3	1.3 ± 0.2	5.0 ± 1.2	7.0 ± 0.5 4.2 ± 0.2
T3RE-2 Region F ME-147/+31 CAT	1.6 ± 0.3	123 ± 32	1.9 ± 0.3	24.1 ± 4.1	70.1 ± 6.8 13.6 ± 2.2
T3RE-2 mut F block ME-147/+31 CAT	3.1±0.3	14.3 ± 4.1	2.2 ± 0.3	7.2 ± 0.3	4.6 ± 1.3 3.4 ± 0.4

Figure 15. A-C/EBP attenuates the stimulation of T3 responsiveness by region F.

Chick embryo hepatocytes were transiently transfected with p[T3RE2]ME-147/+31CAT, p[T3RE2 + Region F]ME-147/+31CAT or p[T3RE2 + mut F block]ME-147/+31CAT in the presence of an A-C/EBP expression plasmid or an empty expression plasmid (RSV vector). After transfection, hepatocytes were treated with or without T3 for 48 h. Cells were then harvested, extracts prepared, and CAT and β-galactosidase assays performed. (Left) constructs used in these experiments. (Right) CAT activity in cells transfected with p[T3RE2]ME-147/+31CAT and treated without T3 was set at 1, and the other activities were adjusted proportionately. The -fold stimulation by T3 was calculated as described in the legend in Fig. 8. The results are the means ± S.E. of 5 experiments.

C/EBPα physically interacts with TR and RXR.

Previous studies have shown that C/EBP can physically interact with the glucocorticoid receptor and modulate the transcription of glucocorticoid responsive genes (Boruk et al., 1998). Data from experiments examining the effects of expression of a dominant negative form of C/EBP on T3 regulation of transcription in CEH confirm that the binding of C/EBP to region F of the malic enzyme gene enhances T3 responsiveness. We hypothesized that the stimulatory effects of C/EBP on T3 responsiveness is mediated, at least in part, by physical interactions between C/EBP bound to region F (-3702 to -3686 bp) and TR/RXR bound to the T3 response unit (-3889 to-3767 bp). To investigate this hypothesis, we determined whether C/EBPα physically interacted with TR and RXR using a pull-down assay. First, we investigated the ability of *in vitro* synthesized TRα and RXRα to bind a bacterially expressed fusion protein containing glutathione S-transferase (GST) linked to C/EBPα. GST-C/EBPα bound ^{35}S-labeled TRα and ^{35}S-labeled RXRα (Fig. 16). The presence of ligand has no effect on the interactions between GST-C/EBPα and nuclear hormone receptors. These data indicate that C/EBPα can interact with both TR and RXR of the TR-RXR heterodimeric complex. These results contrast with data demonstrating that Pbx1 interacts only with TR of the TR-RXR heterodimeric complex (Fig. 10). These variations in the specificity of protein-protein interactions suggest that the protein motifs mediating interactions between TR-RXR and C/EBPα are different from those mediating the interactions between TR-RXR and Pbx1.

Pull-down assays were also carried out using GST-TRα or GST-RXRα as the bait protein. Consistent with results of experiments employing GST-C/EBPα as a bait protein, ^{35}S-labeled C/EBPα strongly interacted with both GST-TRα and GST-RXRα (data not shown). These data further demonstrate that C/EBPα interacts with TRα and RXXα in solution.

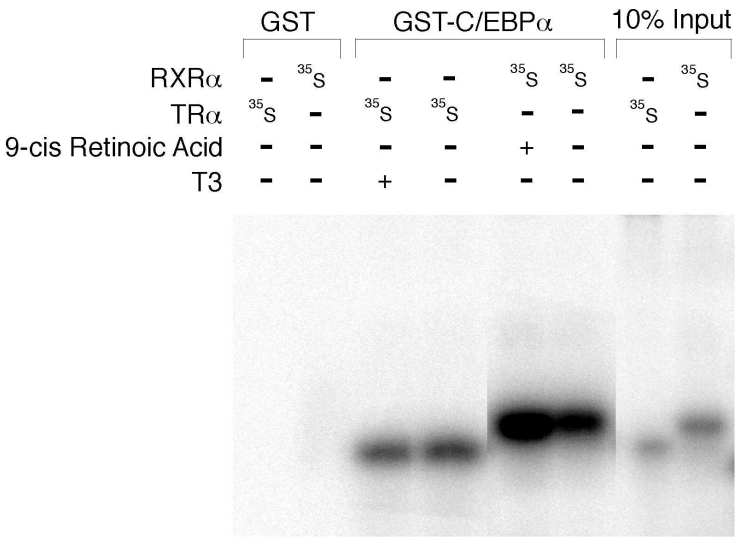

Figure 16. C/EBPα physically interacts with TRα and RXRα.

Bacterially produced GST or GST-C/EBPα immobilized on glutathione-sepharose beads was incubated with *in vitro* translated and ^{35}S-labeled TRα or RXRα as described in "Experimental Procedures*"*. Incubations were performed in the absence and presence of 1 μM T3 or 1 μM 9-cis retinoic acid as indicated. After the matrix was extensively washed, labeled proteins retained on the beads were eluted, resolved by SDS-PAGE, and visualized by storage phosphor autoradiography together with 10% of the total radiolabeled receptor input used in each binding reaction. These data are representative of three experiments.

To map the motifs in TRα that interact with C/EBPα, pull-down experiments were conducted using various truncations of TRα labeled with ^{35}S *in vitro*. Deletion of the first 20 amino acids from the N-terminus of TRα had little or no effect on the binding of TRα to GST-C/EBPα (Fig. 17). Further deletion to amino acid 51 decreased the binding of TRα to GST-C/EBPα. When deletion of the N-terminus of TRα was extended to amino acid 120, binding of TRα to GST-C/EBPα was abolished. To further analyze the interaction between TRα and C/EBPα, TRα polypeptides containing amino acids 1 to 157, amino acids 1 to 118, and amino acids 51 to 157 were tested for their ability to interact with GST-C/EBPα. A strong interaction was observed between GST-C/EBPα and a TRα polypeptide containing amino acids 1 to 157. Markedly weaker but detectable interactions were observed between GST-C/EBPα and TRα polypeptides containing amino acids 1 to 118 and amino acids 51 to 157. Collectively, these data suggest that a TRα region containing the DNA binding domain plus additional flanking sequences (amino acids 21 to 157) is required for optimal binding to C/EBPα. Interestingly, this region of TRα is also required for optimal binding of Pbx1a (Fig. 12).

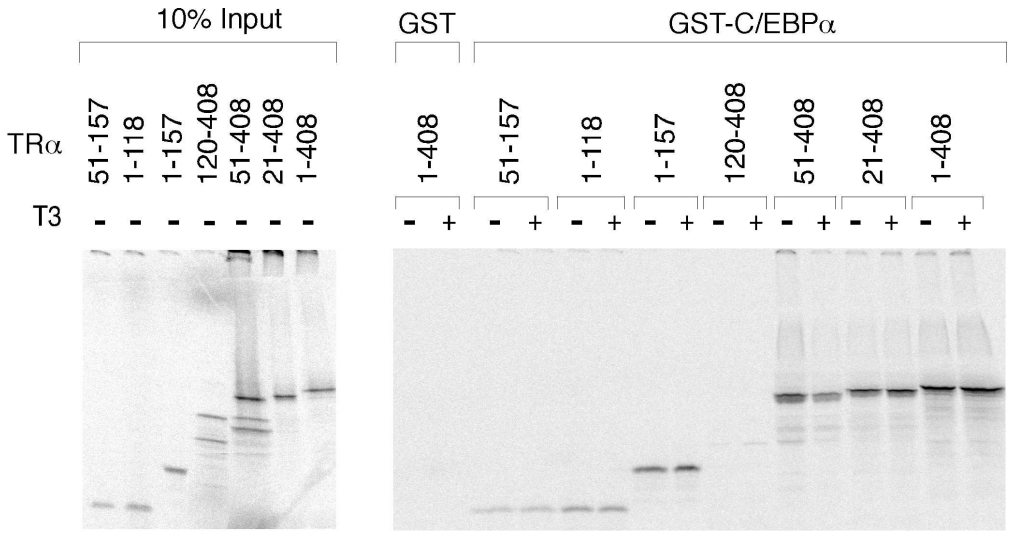

Figure 17. C/EBPα interacts with the DNA binding domain of TRα.

Wild-type and truncated forms of TRα labeled with ^{35}S by *in vitro* translation were incubated with GST or GST-C/EBPα immobilized on glutathione-sepharose beads. Incubations were carried out in the absence and presence of T3 (1 μM). After the matrix was extensively washed, labeled proteins retained on the beads were eluted, resolved by SDS-PAGE, and visualized by storage phosphor autoradiography together with 10% of the total radiolabeled protein input used in each binding reaction.

D. Discussion

Previous studies have shown that C/EBP can functionally and physically interact with other transcription factors to regulate gene transcription. For example, C/EBPα or C/EBPβ interacts with activating transcription factor-2 (ATF-2) and augments transcription directed by various cyclic AMP response elements (CRE) (Haas et al., 1995). C/EBP family members also interact with glucocorticoid receptors causing a stimulation of transcription of the gene for tyrosine aminotransferase (Grange et al., 1991). Moreover, C/EBPβ interacts with the estrogen receptor to mediate the inhibitory effects of estrogen on expression of interleukin 6 gene (Stein and Yang, 1995). The data of the present study are the first to demonstrate a functional and physical interaction between C/EBP and TR-RXR in the regulation of lipogenic gene transcription.

C/EBPα and C/EBPβ have been shown to mediate the stimulatory effects of cAMP on transcription of the genes for phosphoenolpyruvate carboxykinase (Park et al, 1996; Park et al., 1993) and acetyl-CoA carboxylase (Tae et al., 1995). The fact that C/EBP is a regulator of several genes involved in carbohydrate and lipid metabolism has lead to the proposal that this transcription factor plays a central role in the control of energy metabolism (McKnight et al., 1989). Indeed, C/EBPα knockout mice are defective in their ability to maintain blood glucose level and to accumulate lipid in liver and adipose tissue (Wang et al., 1995). Our findings demonstrating that C/EBP is involved in controlling the T3 responsiveness of the malic enzyme promoter provide further support for the hypothesis that this family of transcription factors plays an important role in the regulation of lipogenesis.

Chapter 4. Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) Enhances Thyroid Hormone Responsiveness of the Malic Enzyme Gene in Hepatocytes

A. Introduction

Malic enzyme catalyzes the $NADP^+$-dependent oxidative decarboxylation of malate to pyruvate and CO_2. Much of the NADPH formed in this reaction is used for fatty acid synthesis (Goodridge, 1968). Like many other lipogenic enzymes, malic enzyme activity is regulated by nutritional state. In starved status, malic enzyme activity is low, refeeding causes a 70-fold increase in enzyme activity (Goodridge, 1968). These nutritionally induced changes in malic enzyme activity can be reproduced quantitatively in chick embryo hepatocytes (CEH). In this cell culture system, the active form of thyroid hormone, 3, 5, 3'-triiodothyronine (T3), is the major hormone to regulate malic enzyme activity. Addition of T3 alone to the culture medium stimulates a 40-fold or greater increase in malic enzyme activity. In addition, malic enzyme activity is stimulated by insulin, corticosterone, peroxisome proliferators, and glucose and inhibited by glucagon and fatty acids in the presence of T3 in the culture medium (Goodridge et al., 1987; Goodridge et al., 1989). Regulation of the malic enzyme activity by nutrients and hormones is mediated primarily by a transcriptional mechanism (Ma et al., 1990; Salati et al., 1991).

An interesting feature of the malic enzyme gene is that it is expressed in most tissues and cell lines but its regulation by nutritional state and T3 is restricted to hepatocytes. In heart, kidney and brain, the malic enzyme expression level is low and nutritional manipulation has no effect on malic enzyme transcription in these tissues. Cell-type dependent regulation of malic enzyme is also observed in cells in culture. T3 stimulates a more than 100-fold increase in malic enzyme transcription in CEH, whereas in chick embryo fibroblasts (CEF) and quail QT6 cells, T3 has little or no effect on malic enzyme transcription (Hillgartner et al., 1992). In cells incubated in the absence of T3, the level of malic enzyme transcription in CEF is about 2-fold

higher than that in CEH. In the presence of T3, however, the rate of malic enzyme transcription is about 20-fold higher in CEH than in CEF. Thus, differences in T3 responsiveness between CEH and CEF are due to alterations in transcription in both the absence and presence of T3.

T3 activates gene transcription by interacting with nuclear T3 receptors (TR). TRs bind T3 response elements (T3RE) of target genes as homodimers or heterodimers with the retinoid X receptor (RXR) (Hallenbeck et al., 1992; Yu et al., 1991; Kliewer et al., 1992). T3REs consist of multiple copies of a hexameric sequence related to a consensus RGGWMA arranged as inverted repeats, everted repeats, direct repeats or as extended single copies of the hexamer (Williams et al., 1991; Baniahmad et al., 1990; Näär et al., 1991; Farsetti et al., 1992). TRs confer T3 regulation to a cis-linked gene, at least in part, by interacting with coactivators and corepressors that lack intrinsic DNA binding activity (Kamei et al., 1996; Onate et al., 1995; Chen and Evans, 1995; Horlein et al., 1995). The T3-induced increase in malic enzyme transcription in CEH is mediated by at least six T3REs (Hodnett et al., 1996). One strongly active and four weakly active T3REs are located at -3883/-3769 bp relative to the transcription start site. This cluster of T3REs is referred to as a T3 response unit (T3RU). Another weakly active T3RE is located about 700 bp downstream of the T3RU. All of the malic enzyme T3REs consist of directly repeated hexameric half-sites separated by a 4 bp spacer (DR4) and bind TR-RXR heterodimers in hepatic nuclear extracts. In addition, each of the T3REs contained in the T3RU confers T3 responsiveness by inhibiting transcription in the absence of T3 and activating transcription in the presence of T3 (Hodnett et al., 1996; Thurmond and Goodridge, 1998). This observation is consistent with the data demonstrating that the unliganded TR represses transcription and that binding of T3 to TR reverses this effect and, in some instances, stimulates transcription above that observed in the absence of T3.

Cis-acting sequences that are distinct from T3REs also play an important role in mediating the effects of T3 on malic enzyme transcription. In previous work, we identified four nonreceptor binding sites in the malic enzyme gene that enhanced T3 regulation of transcription (Fang and Hillgartner, 1998). Each of these T3 accessory elements was functional in CEH but not in CEF, suggesting that they played a role in mediating cell-type dependent differences in T3 responsiveness. Three of the malic enzyme T3 accessory elements immediately flanked the T3RU and were designated regions A (-3895/-3890 bp), E (-3761/-3744 bp), and F (-3703/-3686 bp). Further analyses demonstrated that region E bound the homeodomain proteins, Pbx and

Meis1 and that region F bound the liver-enriched factor, CCAAT/enhancer-binding protein-α (C/EBP-α). The stimulatory effects of region E on T3 responsiveness may be mediated by interactions between TR and Pbx, as these proteins specifically interact with each other in solution.

In hepatocytes, the T3RU and accessory elements A, E, and F overlap with a DNase I hypersensitive region (-3910 to -3640 bp) that is induced by the presence of T3 (Fang and Hillgartner, 1998). The 3' boundary of this DNase I hypersensitive region extends about 50 bp downstream of region F, suggesting the presence of additional T3 regulatory sequences 3' of this accessory element. This supposition is supported by the data from DNase I footprinting analyses indicating the presence of protein binding sites between -3681 and -3666 and between -3655 and -3646 in CEH (Fang and Hillgartner, 1998). In the present study, we analyzed the role of these protein binding sites in modulating thyroid hormone regulation of malic enzyme transcription. We have found that both sites enhance T3 responsiveness in CEH but have no effect on T3 responsiveness in CEF. The -3681/-3666 bp site (designated as region G) contains a single nuclear hormone receptor half-site and binds the orphan receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF). The -3655/-3646 bp site (designated as region H) contains an E-box motif and binds protein complexes of unknown identity.

B. Experimental Procedures

Plasmids - The DNA fragments used to construct reporter plasmids were named by designating the 5' and 3' ends of each fragment relative to the start site of transcription of the malic enzyme gene. p[ME-3921/-3631]ME-147/+31CAT has been previously described (Fang and Hillgartner, 1998). This reporter plasmid contains the malic enzyme T3RU and flanking sequences (-3921 to -3631 bp) linked to the minimal promoter of the malic enzyme gene (-147 to +31 bp). Site directed mutations were introduced into p[ME-3921/-3631]ME-147/+31CAT using a polymerase chain reaction-based strategy (Hoton et al., 1990). p[T3RE2]ME-147/+31CAT was made by subcloning the major malic enzyme T3RE between -3883 to -3858 bp into the BamH I/Sac I site upstream of the minimal promoter in ME-147/+31CAT. Region G (ME-3681/-3666) and region H (ME-3655/-3646) were inserted into the Spe I/Sma I site immediately downstream of the

T3RE2 in p[T3RE2]ME-147/+31CAT to form p[T3RE2+G]ME-147/+31CAT and p[T3RE2+H]ME-147/+31CAT, respectively. Dr. M.-J. Tsai (Baylor College of Medicine) provided the cDNAs for human COUP-TFI and COUP-TFII (also known as ARP1). The cDNA for human RXRα was provided by Dr. R. Evans (Salk Institute). cDNAs for COUP-TFI, COUP-TFII, and RXRα were subcloned into pSV-SPORT1 (GIBCO/BRL). Structures of reporter plasmids and expression plasmids were confirmed by restriction enzyme mapping and nucleotide sequence analyses.

Cell Culture and Transient Transfection - Primary cultures of CEH and CEF were prepared as previously described (Goodridge, 1973; Fang and Hillgartner, 1998) and maintained in serum-free Waymouth medium MD705/1 containing 50 nM insulin (gift from Eli Lilly Corp.) and corticosterone (1 μM). Hepatocytes were incubated at 40°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were transfected using a modification of the method of Baillie et al. (Baillie et al., 1993). Briefly, CEH were isolated as described above and plated on 35 mm dishes. At 18 h of incubation, the medium was replaced with one containing 20 μg LipofectACE (GIBCO/BRL), 3.0 μg of p[ME-3921/-3631]ME-147/+31CAT or an equimolar amount of another reporter plasmid and 0.05 μg of pCMV-β-galactosidase (internal transfection standard). At 42 h of incubation, the transfection medium was replaced with fresh medium with or without T3 (1.5 μM). At 90 h of incubation, CEH were harvested and cell extracts were prepared for CAT (Gorman et al., 1982) and β-galactosidase (Sambrook et al., 1989) measurements. CAT activity was expressed relative to β-galactosidase activity to correct for differences in transfection efficiency between samples. All DNAs used in transfection experiments were purified using the Qiagen endotoxin-free kit.

Gel Mobility Shift Analysis - Nuclear extracts were prepared from CEH incubated with or without T3 for 24 h. Nuclei and nuclear extracts were prepared as described (Dignam et al., 1983) except that the protease inhibitors, leupeptin (0.25 μg/ml), benzamidine (10 mM), and PMSF (0.5 mM) were added to the extraction and dialysis buffers at the indicated concentrations. COUP-TFI, COUP-TFII, and RXRα were translated *in vitro* using the TNT SP6 coupled reticulocyte lysate system (Promega). Incorporation of ^{35}S-methionine into COUP-TFI, COUP-

TFII, and RXRα was measured in parallel reactions in order to assess the relative efficiency of synthesis of the different transcription factors.

Double-stranded oligonucleotides were prepared by combining equal amounts of the complementary single-stranded DNA in a solution containing 10 mM Tris, pH 8.0, 1 mM EDTA followed by heating to 95°C for 2 min, and then cooling to room temperature. The annealed oligonucleotides were labeled by filling in overhanging 5' ends using the Klenow fragment of E. *coli* DNA polymerase in the presence of [α-^{32}P]dCTP. Binding reactions were carried out in 20 µl containing 18 mM HEPES, pH 7.9, 90 mM KCl, 0.18 mM EDTA, 0.45 mM DTT, 18% glycerol (v/v), 0.3 mg/ml bovine serum albumin, and 2 µg of poly (dI/dC). A typical reaction contained 20,000 cpm of labeled DNA and 10 µg of nuclear extract or 2 µg of *in vitro* translated proteins. The reaction was carried out on ice for 60 min. DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 4°C in 0.5 X TBE (45 mM Tris, pH, 45 mM boric acid, 1 mM EDTA). Following electrophoresis, the gels were dried and subject to storage phosphor autoradiography. For competition experiments, unlabeled competitor DNA was mixed with radiolabeled oligomer prior to addition of nuclear extract. For gel supershift experiments, nuclear extracts or *in vitro* translated proteins were incubated with antibodies for 1 h at 0°C prior to addition of the oligonucleotide probe. An antiserum that reacts with COUP-TFI and II was generously provided by Dr. M.-J. Tsai. P. Chambon (Strasbourg, France) kindly provided a monoclonal antibody that recognizes the α, β and γ forms of RXR. Synthetic oligonucleotides that were used as probes or competitors in gel mobility shift assays are listed in Fig. 21 and Fig. 23.

C. Results

Malic enzyme sequences between -3681 and -3666 bp (region G) and between -3655 and -3646 bp (region H) enhance T3 regulation of transcription in CEH but not in CEF.

Previous results from DNase I hypersensitivity and DNase I footprinting analyses (Fang and Hillgartner, 1998) suggested the presence of T3 regulatory sequences immediately 3' of region F in the 5'-flanking DNA of the malic enzyme gene (Fig. 4). These sequences are located between

-3681 and -3666 bp and between -3655 and -3646 bp relative to the transcription start site and are designated as region G and region H, respectively. To determine whether region G and region H modulated T3 responsiveness, transient transfection experiments were performed with reporter constructs containing block mutations in region G (mut G block) or region H (mut H block) in the context of p[ME-3921/-3631]ME-147/+31CAT. Mut G block and mut H block decreased T3 responsiveness by 34 and 43 percent, respectively, in CEH (Fig. 18). The effect of mut G block on T3 responsiveness was due to an increase in promoter activity in the absence of T3 and a decrease in promoter activity in the presence of T3, whereas the effect of mut H block was due only to an increase in promoter activity in the absence of T3. In contrast to these data for CEH, mut G block and mut H block had no effect on T3 responsiveness in CEF. Together, these results suggest that region G and region H play a role in mediating cell-type dependent differences in T3 regulation of malic enzyme transcription. Although mut H block had no effect on T3 responsiveness in CEF, this mutation stimulated a marked increase (greater than 7-fold) in promoter activity in these cells in both the absence and presence of T3. Thus, region H also functions as a T3-insensitive repressor of transcription in CEF.

B

Construct	CEH Relative CAT Activity - T3	CEH Relative CAT Activity + T3	CEH Fold Stimulation by T3	CEF Relative CAT Activity - T3	CEF Relative CAT Activity + T3	CEF Fold Stimulation by T3
-3921 -3631 CAT	1.0	329 ± 28	329 ± 28	1.0	10.4 ± 0.1	10.4 ± 0.1
mut G Block CAT	1.3 ± 0.1	273 ± 39	220 ± 22	1.4 ± 0.1	14.5 ± 1.4	10.0 ± 0.4
mut H Block CAT	2.3 ± 0.4	417 ± 72	189 ± 13	7.5 ± 0.7	77.1 ± 5.0	9.7 ± 0.5

Figure 18. Mutations of region G and region H of the malic enzyme gene inhibit T3 responsiveness in chick embryo hepatocytes but not in chick embryo fibroblasts.

(A) Sequence of wild-type and mutant forms of region G and region H. (B) p[ME-3921/-3631]ME-147/+31CAT or constructs containing block mutations of region G (mut G block) or region H (mut H block) in the context of p[ME-3921/-3631]ME-147/+31CAT were transiently transfected into chick embryo hepatocytes (CEH) and chick embryo fibroblasts (CEF) as described under "Experimental Procedures". All transfections contained CMV-β-galactosidase as a control for transfection efficiency. After the transfection, cells were treated with or without T3 for 48 h. Cells were then harvested, extracts were prepared, and CAT and β-galactosidase assays were performed. (Left) the constructs used in these experiments. Numbers indicate the 5' or 3' boundaries of malic enzyme DNA in nucleotides relative to the start site for transcription. (Right) CAT activity was expressed relative to β-galactosidase activity to correct for differences in transfection efficiency. CAT activity in cells transfected with p[ME-3921/-3631]ME-147/+31CAT and incubated without T3 was set at 1, and the other activities were adjusted proportionately. The -fold stimulation by T3 was calculated by dividing CAT activity in hepatocytes treated with T3 (+T3) by that for hepatocytes not treated with T3 (-T3). The -fold responses were calculated for individual experiments and then averaged. The results are the means ± S.E. of 5 experiments.

Characterization of proteins that bind region G.

Gel mobility shift analyses were performed to characterize the proteins that interacted with region G. Incubation of a labeled oligonucleotide probe containing region G (-3682 to -3861 bp, wt G) with nuclear extracts from CEH and CEF resulted in the formation of three protein-DNA complexes that were designated 1, 2, and 3 in the order of increasing mobility (Fig. 19). The abundance of complex 3 in CEH was substantially higher than that of complexes 1 and 2, whereas, in CEF, the abundance of complexes 1 and 2 was markedly higher than that of complex 3. The presence of a 100-fold molar excess of unlabeled oligonucleotide probe abolished the formation of complexes 1, 2, and 3, indicating that the binding of these protein-DNA complexes was specific.

Region G contains a sequence at -3678/-3673 bp (5'-AGGTCA-3') that perfectly matches the consensus half-site sequence for nuclear hormone receptors. Previous studies have demonstrated that COUP-TFI and the highly related factor ARP1 can bind single nuclear hormone receptor half-sites in liver cells (Schräder et al., 1996). For simplicity we will refer to COUP-TFI and ARP1 as COUP-TF. Antibody supershift experiments were performed to determine whether COUP-TF bound region G. Preincubation of nuclear extracts from CEH or CEF with antibodies against COUP-TF disrupted the formation of complexes 1, 2, and 3 (Fig. 19). However, antibodies against TR and RXR, proteins that are capable of forming heterodimers with COUP-TF (Cooney et al., 1993; Leng et al., 1996), had no effect on the formation of complexes 1, 2, and 3. Thus, complexes 1, 2, and 3 contain COUP-TF but not TR and RXR. Comparison of the mobilities of complexes 1, 2 and 3 with that of a protein-DNA complex containing *in vitro* synthesized TRα and RXRα bound to a T3RE suggested that COUP-TF in complexes 1, 2 and 3 was in the form of a dimer or higher order complex (data not shown).

To confirm that COUP-TF bound region G, gel mobility shift experiments were performed using *in vitro* synthesized proteins. Incubation of the region G probe with *in vitro* synthesized COUP-TFI or ARP1 resulted in the formation of a specific complex whose mobility was identical to that of complex 1 in nuclear extracts of CEH and CEF (Fig. 20). This complex was disrupted by the presence of antibodies against COUP-TF. *In vitro* synthesized RXRα alone or in combination with COUP-TFI or ARP1 had no effect on the pattern or extent of protein

binding to region G. These results further demonstrate that region G binds COUP-TF dimers but not COUP-TF/RXR heterodimers.

To identify the sequences in region G that mediated the binding of COUP-TF complexes, competition experiments were performed with unlabeled oligonucleotides containing 3 bp mutations spanning the -3682/-3862 bp region. Oligonucleotides containing mutations that flanked the half-site motif (e.g. -3681/-3679 bp, mut G1; -3672/-3670 bp, mut G3; and -3667/-3665, mut G4) were effective in competing for the binding of complexes 1, 2, and 3 in CEH and CEF, whereas an oligonucleotide containing a mutation within the half-site motif (e.g. -3677/-3675, mut G2) was not effective in competing for the binding of complexes 1, 2, and 3 (Fig. 21). A similar competition profile was observed for *in vitro* synthesized COUP-TFI bound to region G. These data indicate that the binding of COUP-TF to region G requires the presence of the half-site at -3678/-3673 bp and that sequences flanking this motif have little or no effect on binding activity.

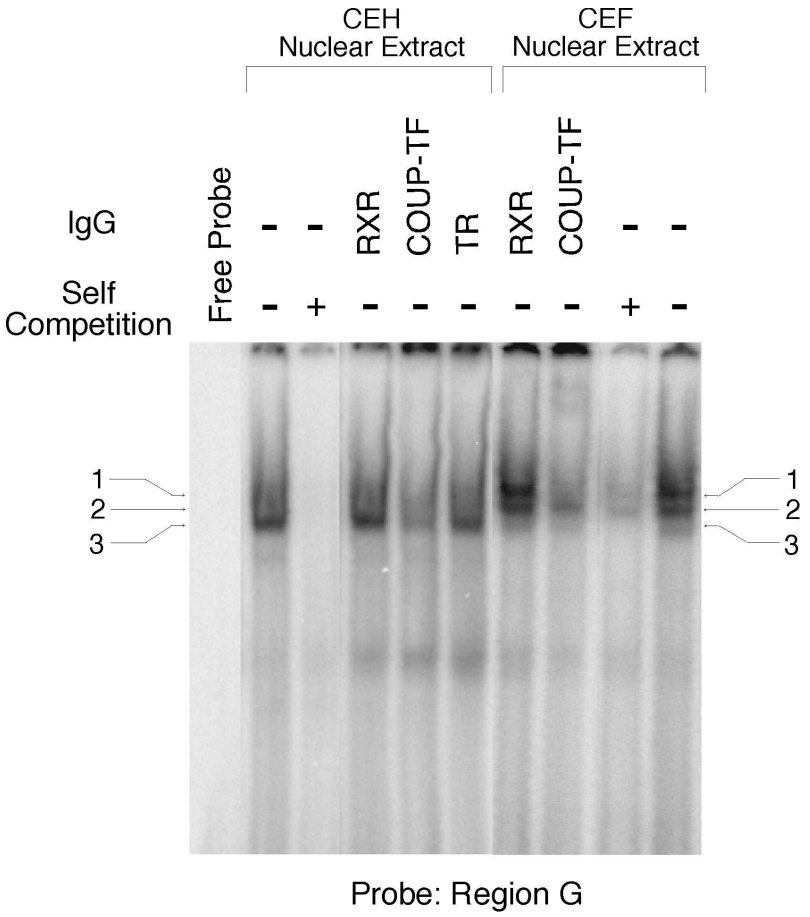

Figure 19. Characterization of proteins that bind region G.

Gel mobility shift experiments were performed using nuclear extracts prepared from chick embryo hepatocytes (CEH) and chick embryo fibroblasts (CEF). A double-stranded DNA fragment containing region G (-3682 to -3862 bp) was labeled with [α-32]dCTP using the Klenow fragment of *E. coli* DNA polymerase. The radiolabeled probe was incubated with 10 μg of nuclear protein in the absence and presence of antibody or a 100-fold molar excess of unlabeled region G (self competition) as described under "Experimental Procedures". DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels. Specific DNA-protein complexes (numbered 1, 2, and 3) are indicated by arrows. These data are representative of four different experiments employing independent preparations of nuclear extract.

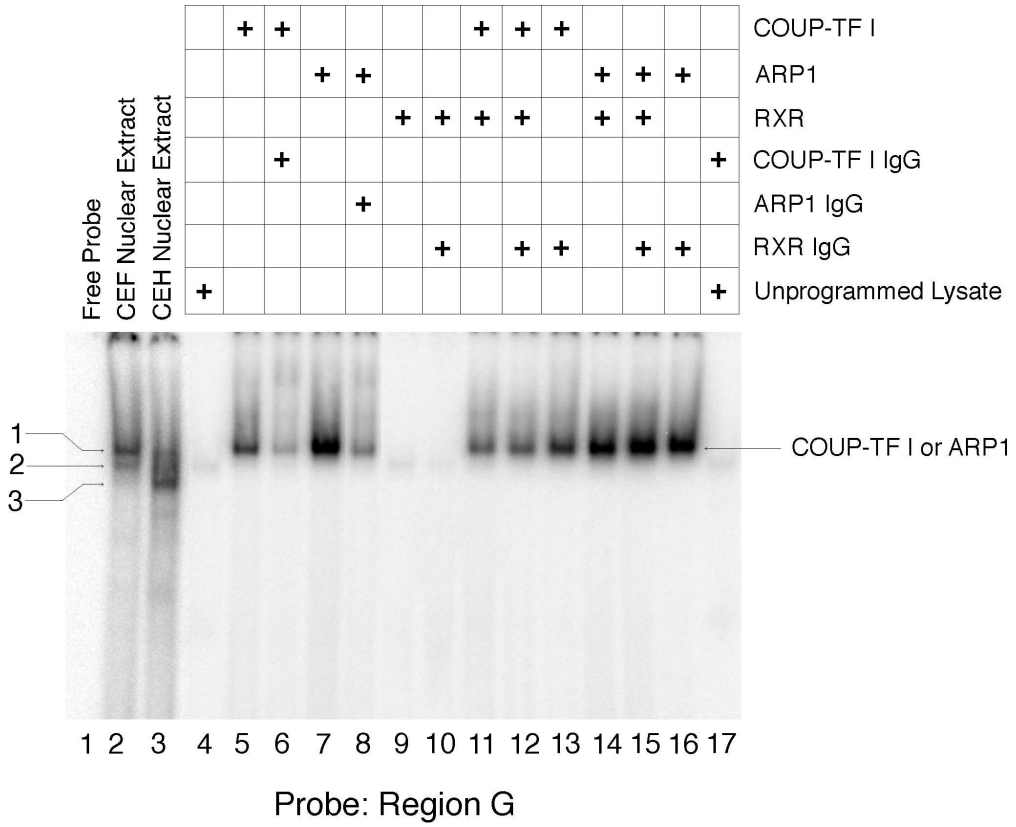

Figure 20. Region G binds *in vitro* synthesized COUP-TFI and ARP1.

Gel mobility shift assays were performed as described under "Experimental Procedures" using a ^{32}P-labeled DNA probe (-3682 to -3862 bp) containing region G. In lanes 5 to 16, equimolar amounts of *in vitro* synthesized COUP-TF-1 or ARP1 were incubated with radiolabeled probe in the absence or presence of *in vitro* synthesized RXR as indicated. In lanes 6, 8, 10, 12, 13, 15, and 16, *in vitro* synthesized proteins were incubated with antibodies against COUP-TF or RXR prior to the addition of the probe. In lane 2 and lane 3, the radiolabeled probe was incubated with nuclear extract from chick embryo hepatocytes (CEH) and chick embryo fibroblasts (CEF), respectively. Positions of complexes 1-3 (lanes 2 and 3) and COUP-TF1 and ARP-1 homodimeric complexes (lanes 5-16) are indicated by arrows.

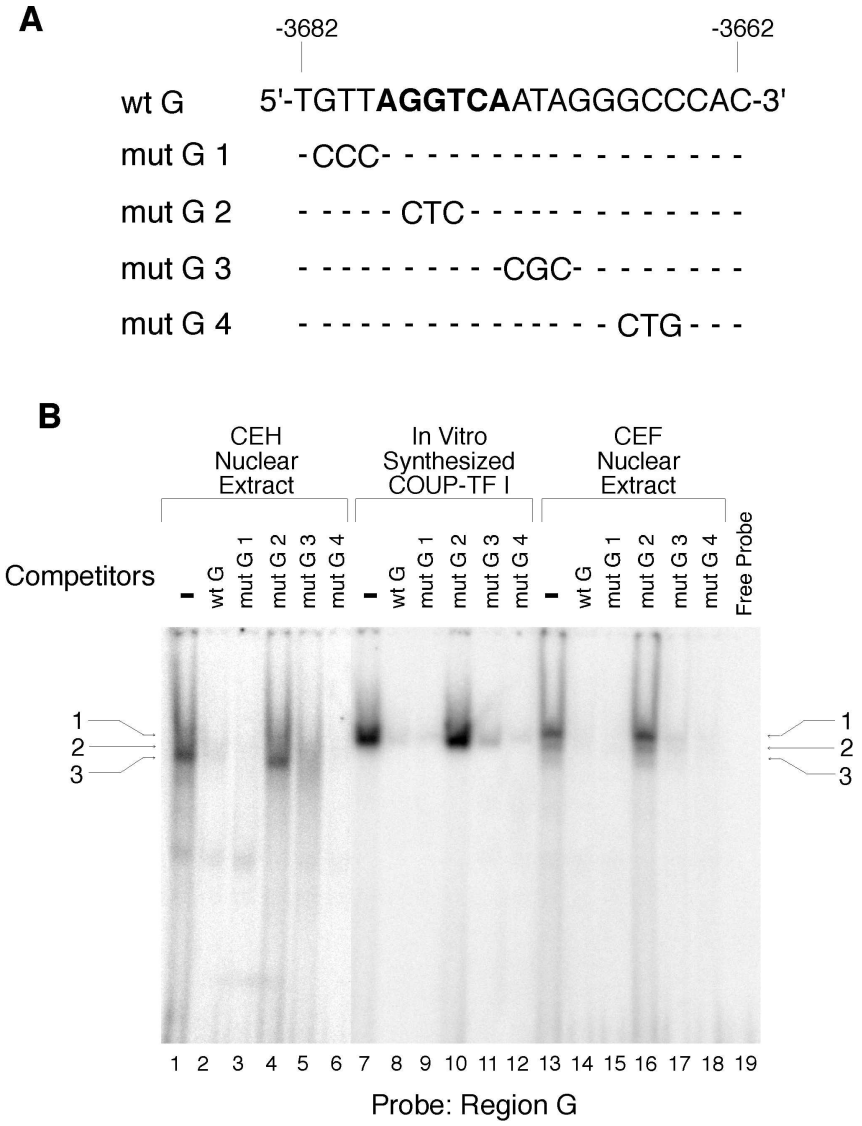

Figure 21. A single nuclear hormone receptor half-site is required for protein binding to region G.

Gel mobility shift experiments were performed as described under "Experimental Procedures" using a ^{32}P-labeled DNA probe (-3682 to -3862 bp) containing region G and chick embryo hepatocytes (CEH) nuclear extract (lanes 1-6), *in vitro* synthesized COUP-TF1 (lanes 7-12), or chick embryo fibroblast (CEF) nuclear extract (lanes 13-18). Incubations with the region G probe were performed in the absence and presence of unlabeled competitor DNAs (50-fold molar excess). DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels. (A) The sequence of the competitor DNAs. wt G is the native malic enzyme sequence between -3682 and -3862 bp. The nuclear hormone receptor half-site contained in wt G is indicated in bold type. mut G1, mut G2, mut G3, and mut G4 contain 3 bp mutations within the -3682 to -3862 bp sequence. (B) Competition analysis with native and mutant competitor DNAs. These data are representative of four experiments employing independent preparations of nuclear extract.

To determine whether binding of COUP-TF to region G was required for optimal T3 responsiveness in CEH, transfection analyses were performed with reporter constructs that contained or lacked a site-specific mutation of the COUP-TF binding site (mut G2) in the context of p[ME-3921/-3631]ME-147/+31CAT. Mut G2 caused a 45% decrease in T3 regulation of promoter activity in CEH (Fig. 22). This observation provides further evidence for a role of COUP-TF complexes in enhancing T3 responsiveness in CEH. Interestingly, mutation of sequences flanking the COUP-TF half-site (mut G1 and mut G4) also inhibited T3 responsiveness in CEH. One explanation for this result is that region G contains binding sites for other transcription factors besides COUP-TF and that the binding of proteins to sites distinct from the COUP-TF binding site is not detectable in the gel mobility shift assay. Alternatively, sequences flanking the COUP-TF binding site may modulate the transcriptional activity of COUP-TF without causing changes in COUP-TF binding affinity. In support of this proposal, previous studies have shown that variations in the structure of the upstream half-site of certain DR4-type T3REs can profoundly alter the transcriptional activity of TR-RXR heterodimers without causing changes in TR-RXR binding affinity (Harbers et al., 1996). The mechanism mediating the alteration in TR-RXR activity involves changes in the conformation of TR-RXR heterodimers bound to DNA (Ikeda et al., 1996).

Characterization of proteins that bind region H.

The binding of proteins to region H was analyzed using the gel mobility shift assay. Incubation of a labeled oligonucleotide probe containing region H (-3663 to -3633 bp, wt H) with nuclear extracts from CEH or CEF resulted in the formation of multiple protein-DNA complexes (Fig. 23). Results from competition analyses with unlabeled oligonucleotides containing sequences that were identical (lanes 2 and 12) or unrelated to the probe sequence (lanes 10 and 20) indicated that marked differences existed between CEH and CEF in the pattern and intensity of sequence-specific protein binding to region H. To determine the sequences in region H that were required for sequence-specific protein binding in CEH and CEF, competition analyses were performed with unlabeled oligonucleotides containing 3 bp mutations spanning the -3663 to -3633 bp region. Oligonucleotide competitors containing mutations at -3659/-3657 bp (mut H1), -3655/-3653 bp (mut H2), -3646/-3644 bp (mut H5), -3643/-3641 bp (mut H6), and -3639/-3637

bp (mut H7) were effective in competing for the binding of sequence-specific protein complexes in CEH and CEF (Fig. 23). In contrast, mutations at -3652/-3650 bp (mut H3) and -3649/-3647 bp (mut H4) were not effective in competing for the binding of sequence-specific protein complexes in CEH and CEF. These data indicate that the region between -3652 and -3647 bp (5'-CAGGTG-3') is required for the binding of sequence-specific protein complexes. This region strongly resembles an E-box motif that binds members of the basic-helix-loop-helix (bHLH) family of transcription factors.

Construct	Relative CAT Activity		Fold Stimulation by T3
	- T3	+ T3	
p[ME-3921/-3631]ME-147/+31CAT	1.0	368 ± 42	368 ± 42
G Block Mutation (-3681/-3666 bp)	1.2 ± 0.1	281 ± 58	222 ± 31
mut G 1 (-3681/-3679 bp)	1.1 ± 0.1	257 ± 44	227 ± 28
mut G 2 (-3677/-3675 bp)	1.0 ± 0.1	206 ± 62	203 ± 44
mut G 4 (-3667/-3665 bp)	1.6 ± 0.2	194 ± 34	132 ± 28
H Block Mutation (-3655/-3646 bp)	2.6 ± 0.1	342 ± 36	134 ± 24
mut H 4 (-3649/-3647 bp)	2.1 ± 0.4	372 ± 118	167 ± 21
mut H 2 (-3655/-3653 bp)	1.7 ± 0.2	299 ± 77	174 ± 27
mut H 5 (-3646/-3644 bp)	1.8 ± 0.1	307 ± 37	169 ± 17

Figure 22. Effects of site-specific mutations within region G and region H on T3 regulation of transcription.

p[ME-3921/-3631]ME-147/+31CAT or constructs containing mutations of region G or region H in the context of p[ME-3921/-3631]ME-147/+31CAT were transiently transfected into chick embryo hepatocytes as described under "Experimental Procedures". Transfections also contained CMV-β-galactosidase as a control for transfection efficiency. After the transfection, cells were treated with or without T3 for 48 h. Cells were then harvested, extracts prepared, and CAT and β-galactosidase assays performed. (Left) the constructs used in these experiments. Numbers indicate the 5' or 3' boundaries of malic enzyme DNA in nucleotides relative to the start site for transcription. The mutated sequences in region G and H are shown in Figs. 18A, 21A, and 23A. (Right) CAT activity was expressed relative to β-galactosidase activity to correct for differences in transfection efficiency. CAT activity in cells transfected with p[ME-3921/-3631]ME-147/+31CAT and incubated without T3 was set at 1, and the other activities were adjusted proportionately. The -fold stimulation by T3 was calculated by dividing CAT activity in hepatocytes treated with T3 (+T3) by that for hepatocytes not treated with T3 (-T3). The -fold responses were calculated for individual experiments and then averaged. The results are the means ± S.E. of 5 experiments.

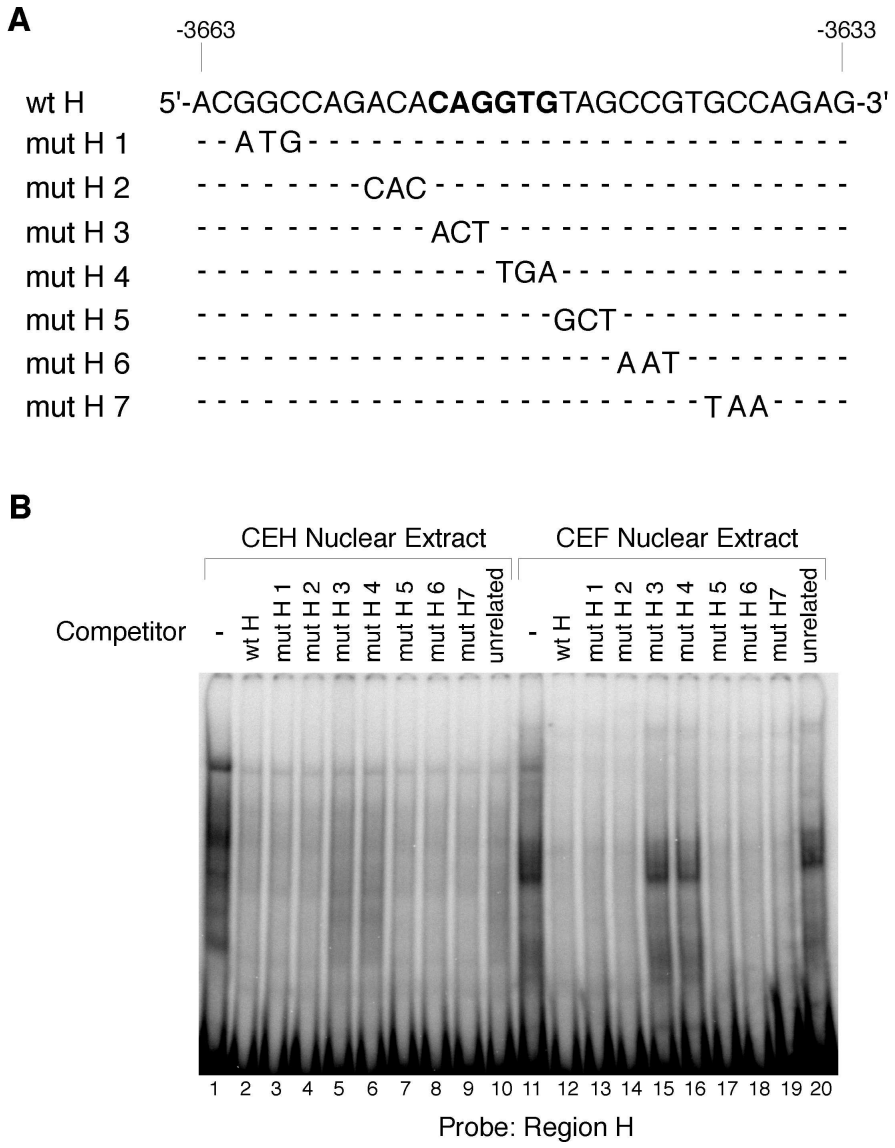

Figure 23. Characterization of proteins that bind to region H using the gel mobility shift assay.

Gel mobility shift experiments were performed as described under "Experimental Procedures" using a ^{32}P-labeled DNA probe (-3663 to -3633 bp) containing region H and nuclear extracts from chick embryo hepatocytes (CEH) (lanes 1-10) and chick embryo fibroblasts (CEF) (lanes 11-20). Incubations with the region H probe were performed in the absence and presence of unlabeled competitor DNAs (50-fold molar excess). DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels. (A) The sequence of the competitor DNAs. wt H is the native malic enzyme sequence between -3663 and -3633 bp. mut H1, mut H2, mut H3, and mut H4, mut H5, mut H6, and mut H7 contain 3 bp mutations within the -3663 to -3633 bp sequence. (B) Competition analysis with native and mutant competitor DNAs. These data are representative of four experiments employing independent preparations of nuclear extract.

In previous work, several bHLH factors have been shown to play a role in regulation of gene transcription in liver. These factors include upstream stimulatory factor-1 (USF-1), upstream stimulatory factor-2 (USF-2), and sterol regulatory element-binding protein-1c (SREBP-1c) (Galibert et al., 1997; Vallet et al., 1997; Shimano et al., 1999). Results from gel mobility shift experiments using antibodies against USF-1, USF-2, or SREBP-1c indicated that none of these factors bound region H in CEH and CEF (data not shown). The lack of binding of USF-1 and USF-2 to region H was supported by the observation that an oligonucleotide containing the consensus binding site for USF was not effective in competing for sequence-specific protein binding to region H in CEH and CEF (data not shown). The sequence of the E-box motif in region H (5'-CAGGTG-3') differs from the sequence of the USF consensus binding (5'-CACGTG-3') site at only one position. Thus, the G residue at position 3 of the E-box motif in region H appears to be an important determinant for sequence-specific protein binding in CEF and CEH.

Transient transfection experiments were performed to determine whether the E-box motif in region H was required for optimal T3 responsiveness in CEH. Mutation of the last 3 bp of the E-box motif (e.g. mut H4) in the context of p[ME-3921/-3631]ME-147/+31CAT caused a 55% decrease in T3 regulation of promoter activity (Fig. 22). This observation provides further support for a role of an E-box binding protein in augmenting T3 responsiveness in CEH transcription. Mutation of sequences flanking the E-box in region H also caused a decrease to a similar level in T3 responsiveness in CEH. These sequences may regulate the transcriptional activity of E-box binding proteins via a mechanism not involving changes in DNA binding affinity.

Region G and region H can function alone to enhance T3 regulation of transcription directed by a T3RE.

Proteins bound to region G and region H may enhance T3 responsiveness in CEH by interacting with TR-RXR heterodimers bound to the adjacent T3RU at -3883/-3769 bp. Alternatively, proteins bound to region G and region H may enhance T3 responsiveness by interacting with other TR accessory proteins bound to sequences flanking the T3RU. To investigate the mechanism by which region G and region H augment T3 regulation of transcription in CEH, transient transfection experiments were conducted to determine whether

region G and region H could function in isolation to enhance T3 regulation of transcription directed by a T3RE. Within the malic enzyme T3RU is a strongly active T3RE designated as T3RE2 (-3883/-3858 bp). When CEH were transfected with a reporter plasmid containing T3RE2 linked to the minimal promoter of the malic enzyme gene (p[T3RE2]ME-147/+31CAT), T3 stimulated a 12.5-fold increase in promoter activity (Fig. 24). When a DNA fragment containing region G (-3682 to -3861 bp) or region H (-3663 to -3633 bp) was inserted downstream of T3RE2 in p[T3RE2]ME-147/+31CAT, the stimulation of promoter activity caused by T3 was increased to more than 20-fold. Insertion of a DNA fragment containing mut G block downstream of T3RE2 in p[T3RE2]ME-147/+31CAT had no effect on T3 responsiveness, indicating that the increase in T3 responsiveness caused by region G and region H was not due to changes in the spacing between T3RE2 and the minimal malic enzyme promoter. These data indicate that region G and region H functionally interact with the TR signaling pathway to enhance T3 regulation of transcription in CEH.

Construct				Relative CAT Activity - T3	Relative CAT Activity + T3	Fold Stimulation by T3
	T3RE-2	ME-147/+31	CAT	1.0	12.5 ± 1.2	12.5 ± 1.2
T3RE-2	Region G	ME-147/+31	CAT	0.6 ± 0.1	12.9 ± 1.9	21.8 ± 1.4
T3RE-2	Region H	ME-147/+31	CAT	0.7 ± 0.1	17.0 ± 2.1	24.2 ± 2.5
T3RE-2	mut G block	ME-147/+31	CAT	1.0 ± 0.2	8.8 ± 1.7	8.9 ± 0.9

Figure 24. Region G and region H can function in isolation to enhance T3 regulation of transcription.

Oligonucleotides containing region G (-3781 to -3662 bp), region H (-3661 to -3633 bp) or a block mutation of region G in the context of the -3781 to -3662 bp fragment (mut G block) were inserted downstream of malic enzyme T3RE2 in p[T3RE2]ME-147/+31CAT. Hepatocytes were transiently transfected with these constructs and treated with or without T3 as described in the legend to Fig. 8. and under "Experimental Procedures". (Left) constructs used in these experiments. (Right) CAT activity in cells transfected with p[T3RE2]ME-147/+31CAT and treated without T3 was set at 1, and the other activities were adjusted proportionately. The -fold stimulation by T3 was calculated as described in the legend in Fig. 8. The results are the means ± S.E. of 5 experiments.

D. Discussion

In this section, we have identified two new TR accessory elements, designated as region G and region H, in an upstream DNase I hypersensitive region of the malic enzyme gene. Region G contains a nuclear hormone receptor half-site that binds the orphan receptor, COUP-TF. Region H contains an E-box motif that interacts with unidentified proteins in CEH and CEF. These proteins appear to be distinct from USF and SREBP. The addition of region G and region H expands the total number of malic enzyme TR accessory elements to five. These accessory elements flank a cluster of five T3REs comprising a T3RU (-3883/-3769 bp) and are arranged 5' to 3' as region A (-3895/-3890 bp), region E (-3761/-3744), region F (-3703/-3686 bp), region G (-3681/-3666 bp), and region H (-3655/-3646 bp) (Fig. 4). Each of these TR accessory elements enhances T3 regulation of transcription in CEH but not in CEF, suggesting that they play a role in mediating cell-type dependent differences in T3 responsiveness. An interesting feature of region H that distinguishes it from the other TR accessory elements is that it functions as a T3-insensitive inhibitor of transcription in CEF. This repressor activity may contribute to the low rate of T3-induced malic enzyme transcription in CEF relative to that observed in CEH (Fang and Hillgartner, 1998).

Previous studies have shown that COUP-TF is a potent repressor of T3 action and that this activity plays an important role in the regulation of brain and muscle development (Tran et al., 1992; Kimura et al., 1993; Klinge et al., 1997). Several mechanisms account for the inhibitory effects of COUP-TF on T3 signaling (Cooney et al., 1993; Leng et al., 1996). COUP-TFs bind to a number of T3REs and, thus, compete with TR for binding to these DNA elements. COUP-TFs may also repress T3 signaling by forming nonproductive complexes with RXR, the heterodimeric partner of TR. In addition, COUP-TFs may repress T3 responsiveness by recruiting corepressors such as nuclear receptor corepressor (N-CoR) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT). The results of the present study are the first to show that COUP-TF can also function as an activator of T3 action. The binding of COUP-TF to region G of the malic enzyme gene may enhance T3 regulation of transcription by interacting with TR bound to the adjacent T3RU. This interaction may facilitate the ability of TR to recruit corepressors in the absence of T3 and coactivators in the presence of T3. COUP-TF may also

enhance T3 regulation of malic enzyme transcription by modulating interactions between TR and the basal transcription factor, TFIIB. Previous studies have shown that both TR and COUP-TF physically interact with TFIIB (Ing et al., 1992). Interactions between COUP-TF and TFIIB and/or between COUP-TF and TR may alter the ability of TR to bind TFIIB in the absence or presence of T3.

Promoter-specific differences in the ability of COUP-TF to activate or inhibit TR activity may be due to variations in the sequences comprising the COUP-TF binding sites. Previous studies have shown that a given DNA sequence can influence the activity of a bound transcription factor via an allosteric mechanism (Hwung et al., 1988; Cooney et al., 1992). The COUP-TF binding site in region G is unusual in that it consists of a single AGGTCA half-site motif rather than two half-sites arranged as direct or inverted repeats. The conformation of COUP-TF bound to region G may facilitate interactions of TR with corepressors in the absence of T3 and coactivators in the presence of T3, whereas the conformation of COUP-TF bound to another element containing a repeated half-site structure may facilitate interactions of TR with corepressors in the absence and presence of T3.

Previous studies have shown that COUP-TF can also function as a positive accessory factor for other hormone signaling pathways. For example, optimal stimulation of the phosphoenolpyruvate carboxykinase (PEPCK) promoter by glucocorticoids is dependent on the presence of two COUP-TF binding sites that flank two glucocorticoid receptor binding sites (Sugiyama et al., 2000). These COUP-TF binding sites, referred to as AF-1 and AF-3, require the presence of at least one other accessory element in the PEPCK promoter in order to be effective in stimulating glucocorticoid responsiveness. The latter observation contrasts with the results of the present study demonstrating that region G can function alone in stimulating hormone responsiveness. Other studies have shown that the retinoic acid-induced stimulation of the retinoic acid receptor-β promoter is augmented by the binding of COUP-TF to a DNA element containing directly repeated half-sites separated by an 8 bp spacer (Lin et al., 2000). COUP-TF stimulates RARβ promoter activity by interacting with RARα bound at an adjacent site. This interaction facilitates the recruitment of CREB binding protein (CBP) to RARα. An interaction between COUP-TF and TR may also mediate the stimulatory effects of region G on T3-induced malic enzyme promoter activity.

Other transcription factors besides COUP-TF and E-box binding proteins can serve as accessory factors for TR. For example, nuclear factor-Y (NF-Y) is required for T3 stimulation of the S14 gene in hepatocytes (Jump et al., 1997). In cardiac muscle cells, myocyte-specific enhancer factor 2 (MEF2) potentiates the ability of TR to stimulate α-cardiac myosin heavy-chain transcription in the presence of T3 (Lee et al., 1997). The α or β isoform of CCAAT/enhancer-binding protein (C/EBP) is required for T3 stimulation of the PEPCK promoter in hepatoma cells (Park et al., 1996; Park et al., 1993). The pituitary-specific DNA binding protein, Pit-1 (also referred to as GHF-1) mediates increased T3 responsiveness of the rat growth hormone promoter in pituitary cells relative to non-pituitary cells (Palomino et al., 1998). Previous work from our laboratory has shown that C/EBP and the homeodomain proteins, Pbx and Meis1 are required for optimal T3 responsiveness of the malic enzyme gene (Chapter 2). Despite the wide diversity of transcription factors that can function as TR accessory proteins, the ability of a given factor to function as a TR accessory protein appears to be dependent on promoter context. For example, AF-1 and AF-3 in the PEPCK promoter are located near a T3RE mediating T3 regulation of PEPCK transcription. Although AF-1 and AF-3 enhance glucocorticoid regulation of promoter activity, they have no effect on T3 regulation of promoter activity (Sugiyama et al., 2000).

Data from gel mobility shift analyses indicate that region G binds multiple protein complexes and that the abundance of these complexes varies between CEH and CEF. We postulate that alterations in protein binding to region G play a role in mediating differences in region G activity between CEH and CEF. In this proposal, complex 3, the predominant complex observed in CEH, would be effective in enhancing T3 responsiveness, whereas complex 1 and complex 2, the predominant complexes observed in CEF, would not be effective in enhancing T3 responsiveness. Complexes 1, 2, and 3 may contain different forms of COUP-TF and/or proteins that dimerize with COUP-TF. Variations in protein binding to DNA may also account for the differences in region H activity between CEH and CEF.

One feature of the malic enzyme gene that distinguishes it from other T3 responsive genes is the large number of T3REs (≥ 5) and TR accessory elements (≥ 5) that mediate T3 regulation of transcription (Hodnett et al., 1996; Fang and Hillgartner, 1998). This feature accounts for the potent effects of T3 on malic enzyme transcription in liver. How the different T3 regulatory sequences cooperate to bring about a high level of T3 responsiveness is presently

unclear. We postulate that TR-RXR heterodimers and TR accessory proteins bound to sequences in DNase I hypersensitive region IV assemble into a stable higher-order nucleoprotein complex referred to as an enhanceosome (Carey, 1998). This enhanceosome may facilitate the assembly of an active RNA polymerase II preinitiation complex on the malic enzyme promoter through direct interactions with coactivators, corepressors, and/or basal transcription factors. Future work will be directed toward defining the protein-DNA and protein-protein interactions controlling enhanceosome function.

Chapter 5. Summary and Future Directions

The promoter of malic enzyme gene contains multiple accessory elements that enhance the T3 regulation of transcription. These elements bind Pbx-Meis1, C/EBP, COUP-TF and an unidentified E-box binding factor. Among these factors, C/EBP is the only one whose expression is liver-specific. Both Pbx-Meis1 and COUP-TF are ubiquitously expressed. We postulate that these accessory factors together form a large multiprotein complex referred as enhanceosome with TR-RXR bound to the T3RU of the malic enzyme gene. The formation of this complex would enhance the ability of TR-RXR to recruit corepressors in the absence of T3 and coactivators in the presence of T3. Corepressors and coactivators would, in turn, function as "bridges" to basal transcriptional machinery via altering the acetylation state of histones or individual components of the basal transcriptional machinery. The cell-type dependent T3 responsiveness may be due to the special role C/EBP plays in the complex, or the cell-type specific posttranslational modification of Pbx, Meis1 and COUP-TF, or the presence of cell-type specific coactivators. The reason why the promoter of malic enzyme gene has such a complicated structure may be due to the fact that malic enzyme is one of genes in which T3 has the most robust stimulatory effect. Multiple accessory proteins are required to achieve the full stimulatory effect by T3. It is also possible that some of those cis-acting elements function to integrate T3 reponsiveness with other signaling pathways such as the response to cAMP and medium chain fatty acids.

The next step in the analysis of the T3 regulation of the malic enzyme gene will be to identify the protein motifs mediating physical and functional interactions of TR-RXR with Pbx-Meis1, COUP-TF, and C/EBP. In addition, the mechanism by which these interactions result in changes in T3 regulation of transcription will be investigated. Here, experiments will be performed to determine whether interactions between TR-RXR and accessory factors modulate the ability of TR to recruit coactivators and corepressors to the malic enzyme gene. Last, the effects of inhibiting the activities of Pbx-Meis1 and COUP-TF on T3 regulation of malic enzyme transcription will be determined to further verify the role of these transcription factors in the control of T3 action.

Chapter 6. References

Abramovich, C., Shen, W. F., Pineault, N., Imren, S., Montpetit, B., Largman, C., and Humphries, R. K. (2000) Functional cloning and characterization of a novel nonhomeodomain protein that inhibits the binding of PBX1-HOX complexes to DNA. *J. Biol. Chem*. **275**, 26172-26177.

Agre, P., Johnson, P. F., and McKnight, S. L. (1989). Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. *Science* 246, 922-926.

Asahara, H., Dutta, S., Kao, H.-Y., Evans, R. M., and Montminy, M. (1999). Pbx-Hox heterodimers recruit coactivator-corepressor complexes in an isoform-specific manner. *Mol. Cll. Biol.* **19**, 8219-8225.

Baillie, R. A., Klautky, S. A., and Goodridge, A. G. (1993). Transient transfection of chick embryo hepatocytes. *J. Nutr. Biochem.* **4**, 431-439.

Baniahmad, A., Ha, I., Reinberg, D., Tsai, S., Tsai, M-J., and O'Malley, B.W. (1993). Interaction of human thyroid hormone receptor with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8832-8836.

Baniahmad, A., Köhne, A. C., and Renkawitz, R. (1992). A transferable silencing domain is present in the thyroid hormone receptor, in the v-*erb*A oncogene product and in the retinoic acid receptor. *EMBO J.* **11**, 1015-1023.

Baniahmad, A., Stein, C., Köhne, A. C., and Renkawitz, R. (1990). Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. *Cell.* **61**, 505-514.

Beckmann, H., Su, L. K., and Kadesch, T. (1990). TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer muE3 motif. *Genes Dev*. **4**, 167-179.

Berthelsen, J., Zappavigna, V., Mavilio, F., and Blasi, F. (1998). Prep1, a novel functional partner of Pbx proteins. *EMBO J.* **17**, 1423-1433.

Bischof, L., Kagawa, N., Moskow, J. J., Takahashi, Y., Iwamatsu, A., Buchberg, A. M., and Waterman, M. R. (1998). Members of the meis1 and pbx homeodomain protein families cooperatively bind a cAMP-responsive sequence (CRS1) from bovine CYP17. *J. Biol. Chem*. **273**, 7941-7948.

Bogazzi, F., Hudson, L.D., and Nikodem, V.M. (1994) A novel heterodimerization partner for thyroid hormone receptor. *J. Biol. Chem.* **269**, 11683-11688.

Boruk, M., Savory, J. G., and Hache, R. J. (1998). AF-2-dependent potentiation of CCAAT enhancer binding protein beta-mediated transcriptional activation by glucocorticoid receptor. *Mol Endocrinol.* **12**, 1749-1763.

Bouchard, C., Staller, P., and Eilers, M. (1998). Control of cell proliferation by Myc. *Trends Cell Biol.* **8**, 202-206.

Burglin, T. (1997). Analysis of TALE superclass homeobox genes reveals a novel domain conserved between plants and animals. *Nucleic Acids Res*. **25**, 4173-4180.

Carey, M. (1998). The enhanceosome and transcriptional synergy. *Cell.* **92,** 5-8.

Calkhoven, C. F., Ab, G., Wijnholds, J. (1992) c/CEPB, a chicken transcription factor of the leucine-zipper C/EBP family. *Nucleic Acids Res*. **20**, 4093.

Chang, C.-P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G., and Cleary, M. L. (1997). Meis proteins are major *in vivo* DNA binding partners for wild-type but not chimeric Pbx proteins. *Mol. Cll. Biol.* **17**, 5679-5687.

Chang, C.-P., Shen, W.-F., Rozenfeld, S., Lawrence, H. J., Largman, C., and Cleary, M. L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev*. **9**, 663-674.

Chen, J. D., and Evans, R. M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377**, 454-457.

Cheng, X., Reginato, M. J., Andrews, N. C., and Lazar, M. A. (1997) The transcriptional integrator CREB-binding protein mediates positive cross talk between nuclear hormone receptors and the hematopoietic bZip protein p45/NF-E2. *Mol. Cell. Biol*. **17**, 1407-1416.

Cheneval, D., Christy, R. J., Geiman, D., Cornelius, P., and Lane, M. D. (1991) Cell-free transcription directed by the 422 adipose P2 gene promoter: activation by the CCAAT/enhancer binding protein. *Proc Natl Acad Sci U. S. A.* **88**, 8465-8469.

Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lan, M. D. (1991). CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. U. S. A*. **88**, 2593-1597.

Cooney, A. J., Leng, X., Tsai, S. Y., O'Malley, B. W., and Tsai, M.-J. (1993). Multiple mechanisms of chicken ovalbumin upstream promoter transcription factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. *J. Biol. Chem.* **268**, 4152-4160.

Cooney, A. J., Tsai, S. Y., O'Malley, B. W., and Tsai, M.-J. (1992). Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. *Mol. Cll. Biol.* **12**, 4153-4163.

Damm, K., Thompson, C. C., and Evans, R. M. (1989). Protein encoded by v-*erb*A functions as a thyroid-hormone receptor antagonist. *Nature* **339**, 593-597.

Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983). Accurate transcription initiation by RXA polymerase II in a soluble extrat from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475-1489.

Disela, C., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H., Zenke, M. (1991). v-*erb*A overexpression is required to extinguish c-*erb*A function in erythroid cell differentiation and regulation of the *erb*A target gene CAII. *Genes Dev.* **5**, 2033-2047.

Dobrovic, A., Houle, B., Belouchi, A., and Bradley, W. E. C. (1988). *erb*A-related sequence coding for DNA-binding hormone receptor localized to chromosome 3p21-3p25 and deleted in small cell lung carcinoma. *Cancer Res.* **48**, 682-685.

Edwards, D. P. (1999) Coregulatory proteins in nuclear hormone receptor action. *Vitam. Horm*. **55**, 165-218.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889-895.

Fang, X., and Hillgartner, F. B. (1998). Cell-specific regulation of transcription of the malic enzyme gene: characterization of cis-acting elements that modulate nuclear T3 receptor activity. *Arch. Biochem. Biophys*. **349**, 138-152.

Farsetti, A., Desvergne, B., Hallenbeck, P., Robbins, J., and Nikodem, V. M. (1991). Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain. *J. Biol. Chem*. **266**, 23226-26232.

Ferretti, E., Schulz, H., Talarico, D., Blasi, F., and Berthelsen, J. (1999). The PBX-regulating protein PREP1 is present in different PBX-complexed forms in mouse. *Mech. Dev.* **83**, 53-64.

Fisher, D. E., Carr, C. S., Parent, L. A., and Sharp P. A. (1991). Myc/Max and other helix-loop-helix/leucine zipper proteins bend DNA toward the minor groove. *Genes Dev*. **5**, 2342-2352.

Flodby, P., Barlow, C., Kylefjord, H., Ahrlund-Richter, L., and Xanthopoulos, K. G. (1996). Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein alpha. *J Biol Chem*. **271**, 24753-24760.

Fondell, J.D., Roy, A.L., and Roeder, R.G. (1993) Unliganded thyroid hormone receptor inhibits formation of a functional preinitiation complex: Implications for active repression. *Genes Dev.* **7**, 1400-1410.

Galibert, M. D., Boucontet, L., Goding, C. R., Meo, T. (1997). Recognition of the E-C4 element from the C4 complement gene promoter by the upstream stimulatory factor-1 transcription factor. *J. Immunol*. **159**, 6176-6183.

Gandrillon, O., Jurdic, P., Benchaibi, M., Xiao, J.-H., Ghysdael, J., and Samarut, J. (1987). Expression of the v-*erb*A oncogene in chicken embryo fibroblasts stimulates their proliferation *in vitro* and enhances tumor growth *in vivo*. *Cell* **49**, 687-697.

Gandrillon, O., Jurdic, P., Pain, B., Desbois, C., Madjar, J. J., Moscovici, M. G., Moscovici, C., and Samarut, J. (1989). Expression of the v-*erb*A product, an altered nuclear hormone receptor, is sufficient to transform erythrocytic cells *in vitro*. *Cell* **58**, 115-121.

Glass, C. K., and Holloway, J. M. (1990). Regulation of gene expression by the thyroid hormone receptor. *Biochim. Biophys. Acta* **1032**, 157-176.

Glass, C.K., Lipkin, S.M., Devary, O.V., and Rosenfeld, M.G. (1989). Positive and negative regulation of gene transcripton by a retinoic acid-thyroid hormone receptor heterodimer. *Cell* **59**, 697-708.

Glass, C.K. and Rosenfeld, M.G. (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev*. **14**, 147-151.

Goodridge, A. G. (1968). The effect of starvation followed by feeding on enzyme activity and the metabolism of [U-14C] glucose in liver. *Biochem. J.* **108**, 667-673.
Goodridge, A. G. (1973). Regulation of fatty acid synthesis in isolated hepatocytes prepared from the livers neonatal chicks. *J. Biol. Chem.* **248**, 1924-1931.

Goodridge, A. G. (1987). Dietary regulation of gene expression: enzymes involved in carbohydrate and lipid metabolism. *Annu. Rev. Nutr*. **7**, 157-185.

Goodridge, A. G., and Adelman, T. G. (1976). Regulation of malic enzyme synthesis by insulin, triiodothyronine, and glucagon in liver cells in culture. *J. Biol. Chem*. **251**, 3027-3032.

Goodridge, A. G., Crish, J. F., Hillgartner, F. B., and Wilson, S. B. (1989). Nutritional and hormonal regulation of the gene for avian malic enzyme. *J. Nutr*. **119**, 299-308.

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cll. Biol.* **2**, 1044-1051.

Goudet, G., Delhalle, S,. Biemar, F., Martial, J. A., Peers, B. (1999). Functional and cooperative interactions between the homeodomain PDX1, Pbx and Prep1 factors on the somatostatin promoter. *J. Biol. Chem*. **274**, 4067-4073.

Graf, T., and Beug, H. (1983). Role of v-*erb*A and v-*erb* B oncogenes of avian erythroblastosis virus in erythroid cell transformation. *Cell* **34**, 7-9.

Grange, T., Roux, J., Rigaud, G., and Pictet, R. (1991). Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor. *Nucleic Acids Res*. **19**, 131-139.

Haas, N. B., Cantwell, C. A., Johnson, P. F., and Burch, J. B. (1995). DNA-binding specificity of the PAR basic leucine zipper protein VBP partially overlaps those of the C/EBP and CREB/ATF families and is influenced by domains that flank the core basic region. *Mol Cell Biol*. **15**, 1923-1932.

Hadzic, E., Desai-Yajnik, V., Helmer, E., Guo, S., Wu, S., Koudinova, N., Casanova, J., Raaka, B. M., and Samuels, H. H. (1995). A 10-amino-acid sequence in the N-terminal A/B domain of thyroid hormone receptor alpha is essential for transcriptional activation and interaction with the general transcription factor TFIIB. *Mol. Cll. Biol.* **15**, 4507-4517.

Hallenbeck, P. L., Marks, M. S., Lippoldt, R. E., Ozato, K., and Nikodem, V. M. (1992). Heterodimerization of thyroid hormone (TH) receptor with H-2RIIBP (RXR beta) enhances DNA binding and TH-dependent transcriptional activation. *Proc. Natl. Acad. Sci. U. S. A*. **89**, 5572-5576.

Harbers, M., Wahlstrom, G. M., Vennstrom, B. (1996). Transactivation by the thyroid hormone receptor is dependent on the spacer sequence in hormone response elements containing directly repeated half-sites. *Nucleic Acids Res*. **24**, 2252-2259.

Herzig, S., Fuzesi, L., Knepel, W. (2000). Heterodimeric Pbx-Prep1 homeodomain protein binding to the glucagon gene restricting transcription in a cell type-dependent manner. *J. Biol. Chem*. **275**, 27989-27999.

Hillgartner, F. B., Charron, T. (1998). Glucose stimulates transcription of fatty acid synthase and malic enzyme in avian hepatocytes. *Am J Physiol*. **274** (3 Pt 1), E493-501.

Hillgartner, F.B., Charron, T., and Chesnut, K.A. (1996). Alterations in nutritional status regulate acetyl-CoA carboxylase expression in avian liver by a transcriptional mechanism. *Biochem. J.* **319**, 263-268.

Hillgartner, F. B., Chen, W., and Goodridge, A. G. (1992). Overexpression of the α-thyroid hormone receptor in avian cell lines. Effects on expression of the malic enzyme gene are selective and cell-specific. *J. Biol. Chem.* **267**, 12299-12306.

Hillgartner, F. B., and Romsos, D. R. (1987). Nuclear triiodothyronine receptor binding characteristics and occupancy in obese (ob/ob) mice. *Am. J. Physiol.* **252** (3 Pt 1), E414-E419.

Hillgartner, F. B., Salati, L. M., and Goodridge, A. G. (1995). Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. *Physiol. Rev*. **75**, 47-76.

Hodnett, D.W., Fantozzi, D.A., Thurmond, D.C., Klautky, S.A., MacPhee, K.G., Estrem, S.T., Xu, G., and Goodridge, A.G. (1996). The chicken malic enzyme gene. Structural organization and identification of the triiodothyronine response elements in the 5'-flanking DNA. *Archives of Biochem. Biophys.* **334**, 309-324.

Horlein, A.J., Naar, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C.K. and Rosenfeld, M.G. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377**, 397-404.

Horton, R. M., Cai, Z., Ho, S. N., and Pease, L. R. (1990). Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* **134**, 528-535.

Hwung, Y.-P., Wang, L.-H., Tsai, S. Y., and Tsai, M.-J. (1988). Differential binding of the chicken ovalbumin upstream promoter (COUP) transcription factor to two different promoters. *J. Biol. Chem.* **263**, 13470-13474.

Ikeda, M., Kawaguchi, A., Takeshita, A., Chin, W. W., Endo, T., and Onaya, T. (1999). CBP-dependent and independent enhancing activity of steroid coactivator-1 in thyroid hormone receptor-mediated transactivation. *Mol. Cell. Endocrinol*. **147**, 103-112.

Ikeda, M., Wilcox, E. C., Chin, W. W. (1996). Different DNA elements can modulate the conformation of thyroid hormone receptor heterodimer and its transcriptional activity. *J. Biol. Chem*. **271**, 23096-23104.

Ing, N. H., Beekman, J. M., Tsai, S. Y., Tsai, M. J, AND O'Malley, B. W. (1992). Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). *J. Biol. Chem*. **267**, 17617-17623.

Jacobs, Y., Schnabel, C. A., and Cleary, M. L. (1999). Trimeric association of Hox and TALE homeodomain proteins mediates Hoxb2 hindbrain enhancer activity. *Mol. Cell. Biol.* **19**, 5134-5142.

Johnson, P. F., Landschulz, W. H., Graves, B. H., and McKnight, S. L. (1987). Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev*., **1**, 133-146.

Jump, D.B., Badin, M.V., and Thelen, A. (1997) The CCAAT box binding factor, NF-Y, is required for thyroid hormone regulation of rat liver S14 gene transcription. *J. Biol. Chem.* **272**, 27778-27786.

Kaestner, K. H., Christy, R. J., and Lane, M. D. (1990). Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci. U. S. A*. **87**, 251-255.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K., and Rosenfeld, M.G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403-414.

Kamps, M. P., Murre, C., Sun, X.-H., and Baltimore, D. (1990). A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. *Cell* **60**, 547-555.

Kastner, P. M., Mark, M., and Chambon, P. (1995). Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* **83**, 859-860.

Katz, K., Kowenz-Leutz, E., Muller, C., Meese, K., Ness, S. A., and Leutz, A. (1993) The NF-M transcription factor is related to C/EBPβ and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. *EMBO. J*. **12**, 1321-1332.

Kimura, A., Nishiyoti, A., Murakami, T. Tsukamoto, T., Hata, S., Osumi, T., Okamura, R., Mori, M., and Tasiguchi, M. (1993). Chicken ovalbumin upstream promoter-transcriptin factor represses transcription from the promoter of the gene for ornithine transcarbamylase in a manner antagonistic to hepatocyte nuclear factor-4 (HNF-4). *J. Biol. Chem.* **268**, 11125-11133.

Kliewer, S.A., Umesono, K., Mangelsdorf, D.J., and Evans, R.M. (1992). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. *Nature* **355**, 446-449.

Klinge, C., Silver, B. F., Driscoll, M. D., Sathya, G., Bambara, R., and Hilf, R. (1997). Chicken ovalbumin upstream promoter-transcription factor interacts with estrogen receptor, binds to estrogen response elements and half-sites, and inhibits estrogen-induced gene expression. *J. Biol. Chem.* **272**, 31465-31474.

Knoepfler, .P. S., Bergstrom, D. A., Uetsuki, T., Dac-Korytko, I., Sun, Y. H., Wright, W. E., Tapscott, S. J., and Kamps, M. P. (1999). A conserved motif N-terminal to the DNA-binding domains of myogenic bHLH transcription factors mediates cooperative DNA binding with pbx-Meis1/Prep1. *Nucleic Acids Res*. **27**, 3752-3761.

Knoepfler, P. S., Calvo, K., R., Chen, H., Antonarakis, S. E., and Kamps, M. P. (1997). Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a-Pbx1. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14553-14558

Kruse, F., Rose S. D., Swift, G. H., Hammer, R. E., and MacDonald, R. J. (1995). Cooperation between elements of an organ-specific transcriptional enhancer in animals. *Mol. Cell. Biol*. **15**, 4385-4394.

Ktistaki, E., and Talianidis, I. (1997). Chicken ovalbumin upstream promoter transcription factors act as auxiliary cofactors for hepatocyte nuclear factors and enhance hepatic gene expression. *Mol. Cell. Biol.* **17**, 2790-2797.

Lazar, M. A., Berrodin, T. J., and Harding, H. P. (1991). Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor. *Mol. Cell. Biol.* **11**, 5005-5015.

Lazar, M. A., and Chin, W. W. (1990). Nuclear thyroid hormone receptors. *J. Clin. Invest.* **86**, 1777-1782.

Lazennec, G., Kern, L., Valotaire, Y., and Salbert, G. (1997). The nuclear orphan receptors COUP-TF and ARP-1 positively regulate the trout estrogen receptor gene through enhancing autoregulation. *Mol. Cell. Biol.* **17**, 5053-5066.

Lee, Y., Nadal-Ginard, B., Mahdavi, V., and Izumo, S. (1997). Myocyte-specific enhancer factor 2 and thyroid hormone receptor associate and synergistically activate the alpha-cardiac myosin heavy-chain gene. *Mol. Cell. Biol*. **17**, 2745-2755.

Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., and Chambon, P. (1992). Purification, cloning, and RXR identity of HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68**, 377-395.

Leng, X., Cooney, A. J., Tsai, S. Y., and Tsai, M.-J. (1996). Molecular mechanisms of COUP-TF-mediated transcriptional repression: evidence for transrepression and active repression. *Mol. Cell. Biol.* **16**, 2332-2340.

Lin, B., Chen, G. Q., Xiao, D., Kolluri, S. K., Cao, X., Su, H., Zhang, X. K. (2000). Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition, and apoptosis by retinoic acid in cancer cells. *Mol. Cell. Biol*. **20**, 957-970.

Lin, F., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993). A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc. Natl. Acad. Sci. U. S. A*. **90**, 9606-9610.

Lumsden, A., and Krumlauf, R. (1996). Patterning the vertabrate neuraxis. *Science* **274**, 1109-1115.

Ma, X.-J., Salati, L. M., Ash, S. E., Metchell, D. A., Klautky, S. A., Fantozzi, D. A., and Goodridge, A. G. (1990). Nutritional regulation and tissue-specific expression of the malic enzyme gene in the chicken. Transcriptional control and chromatin structure. *J. Biol. Chem*. **265**, 18435-18441.

MacGregor, G. R., and Caskey, C. T. (1989). Construction of plasmids that express *E. coli* beta-galactosidase in mammalian cells. *Nucleic Acids Res*. **17**, 2365.

Malik, S., and Karathanasis, S. (1995). Transcriptional activation by the orphan nuclear receptor ARP-1. *Nucleic Acids Res.* **23**, 1536-1543.

Mangelsdorf, D. J., and Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. *Cell* **83**, 835-839.

Mangelsdorf, D. J., and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83**, 841-850.

Marcus, Sl, L., Winrow, C. J., Capone, J. P., and Rachubinski, R. A. (1996). A p56lck ligand serves as a coactivator of an orphan nuclear hormone receptor. *J. Biol. Chem.* **271**, 27197-27200.

McKnight, S. L., Lane, M. D., and Glueckshon-Waelch, S. (1989). Is CCAAT/enhancer-binding protein a central regulator of energy metabolism? *Genes Dev*. **3**, 2021-2024.

Monica, K., Galili, N., Nourse, J., Saltman, D., and Cleary, M. (1991). PBX2 and PBX3, new homeobox genes with extensive homology to the human proto-oncogene PBX1. *Mol. Cell. Biol.* **11**, 6149-6157.

Moore, R., Mehrishi, J. N., Verdoorn, C., and Mills, I. H., (1981). The role of T3 and its receptor in efficient metabolisers receiving very-low-calorie diets. *Int. J. Obesity* **5**, 283-286.
Näär, A. M., Boutin, J. M., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K., and Rosenfeld, M. G. (1991). The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell*. **65**, 1267-1279.

Nerlov, C., and Ziff, E. B. (1994). CCAAT/enhancer binding protein-alpha amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cells. *EMBO J*. **14**, 4318-4328.

Nerlov, C., and Ziff, E. B. (1995). Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-alpha on the serum albumin promoter. *Genes Dev*. **8**, 350-362.

Nishio, Y., Isshiki, H., Kishimoto, T., and Akira, S. (1993). A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat 1-acid glycoprotien gene via direct protein-protein interaction. *Mol. Cell. Biol*. **13**, 1854-1862.

Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S., and Cleary M. (1990). Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. *Cell* **60**, 535-545.

Ogo, A., Waterman, M., McAllister, J. M., and Kagawa, N. (1997). The homeodomain protein Pbx1 is involved in cAMP-dependent transcription of human CYP17. *Arch. Biochem. Biophys*. **348**, 226-231.

Onate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**,1354-1357.

Palomino, T., Sanchez-Pacheco, A., Pena, P., and Aranda, A. (1998). A direct protein-protein interaction is involved in the cooperation between thyroid hormone and retinoic acid receptors and the transcription factor GHF-1. *FASEB J.* **12**, 1201-1209.

Park, E. A., Gurney, A. L., Nizielski, S. E., Hakimi, P., Cao, Z., Moorman, A., and Hanson, R. W. (1993). Relative roles of CCAAT/enhancer-binding protein beta and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP). *J. Biol. Chem.* **267**, 613-619.

Park, E. A., Jerden, D.C., and Bahouth, S.W. (1995). Regulation of phosphoenolpyruvate carboxykinase gene transcription by thyroid hormone involves two distinct binding sites in the promoter. *Biochem. J.* **309**, 913-919.

Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J., Friedman, A., and Hanson, R. W. (1990). The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol Cell Biol*. **10**, 6264-6272.

Park, E. A., Song, S., Oliver, M., and Roesler, W. J. (1997). CCAAT-enhancer-binding protein alpha (C/EBP alpha) is required for the thyroid hormone but not the retinoic acid induction of phosphoenolpyruvate carboxykinase (PEPCK) gene transcription. *Biochem. J.* **322** (Pt 1), 343-349.

Park, E.A., Song, S., Vinson, C., and Roesler, W. J. (1999). Role of CCAAT enhancer-binding protein beta in the thyroid hormone and cAMP induction of phosphoenolpyruvate carboxykinase gene transcription. *J. Biol. Chem*. **274**, 211-217.

Peers, B., Sharma, S., Johnson, T., Kamps, M., and Montminy, M. (1995). The pancreatic islet factor STF-1 binds cooperatively with Pbx to a regulatory element in the somatosatin promoter: importance of the FPWMK motif and of the homeodomain. *Mol. Cell. Biol.* **15**, 7091-7097.

Penkov, D., Tannaka, S., Ricco, G. D., Berthelsen, J., Blasi, F., and Ramirez, G. (2000). Cooperative interactions between PBX, PREP, and HOX proteins modulate the activity of the alpha 2(V) collagen (COL5A2) promoter. *J. Biol. Chem*. **275**. 16681-16689.

Pereira, F. A., Qiu, Y., Tsai, M. J., Tsai, S. Y.(1995). Chicken ovalbumin upstream promoter transcription factor (COUP-TF): expression during mouse embryogenesis. *J. Steroid Biochem. Mol Biol*. **53**, 503-508.

Popperl, H., Rikhof, H., Chang, H., Haffter, P., Kimmel, C. B., and Moens, C. B. (2000) lazarus is a novel pbx gene that globally mediates hox gene function in zebrafish. *Mol Cell*. **6**, 255-267.

Reginato, M.J., Zhang, J., and Lazar, M.A. (1996). DNA-independent and DNA-dependent mechanisms regulate the differential heterodimerization of the isoforms of the thyroid hormone receptor with retinoid X receptor. *J. Biol. Chem.* **271**, 28199-28205.

Richie, H. H., Wang L.-H., Tsai, S., O'Malley, B. W., and Tsai, M.-J. (1990). COUP-TF gene: a structure unique for the steroid/thyroid receptor superfamily. *Nucleic Acids Res.* **18**, 6857-6862.

Sagami, I., Tsai, S. Y., Wang, H., Tsai, M.-J. and O'Malley, B. W. (1986). Identification of two factors required for transcription of the ovalbumin gene. *Mol. Cell. Biol.* **6**, 4259-4267.

Salati, L. M., Ma, X-J., McCormick, C.C., Stapleton, S. R., and Goodridge, A. G. (1991). Triiodothyronine stimulates and cyclic AMP inhibits transcription of the gene for malic enzyme in chick embryo hepatocytes in culture. *J. Biol. Chem*. **266**, 4010-4016.

Sambrook, J. Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning, a laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sap, J., Muñoz, A., Schmitt, J., Stunnenberg, H., and Vennström, B. (1989). Repression of transcription mediated at a thyroid hormone response element by the v-*erb*A oncogene product. *Nature* **340**, 242-244.

Schaufele, F., West, B.L., and Baxter, J.D. (1992). Synergistic activation of the rat growth hormone promoter by pit-1 and the thyroid hormone receptor. *Mol. Endocrinol*. **6**, 656-665.

Schräder, M., Danielsson, C., Wiesenberg, I., and Carlberg, C. (1996). Identification of natural monomeric response elements of the nuclear receptor RZR/ROR. *J. Biol. Chem.* **271**, 19732-19736.

Shanmugam, K., Green N. C., Rambaldi, I., Saragovi, H. U., and Featherstone, M. S. (1999). PBX and MEIS as non-DNA-binding partners in trimeric complexes with HOX. *Mol. Cell. Biol.* **19**, 7577-7588.

Shen, W.-F., Rozenfeld, S., Kwong, A., Kömüves, L. G., Lawrence, H. J., and Largman, C. (1999). HOXA9 forms triple complexes with PBX2 and MEIS1 in myeloid cells. *Mol. Cell. Biol.* **19**, 3051-3061.

Shibata, H., Nawaz, Z., Tsai, S. Y., O'Malley, B. W., and Tsai, M.-J. (1997). Gene silencing by chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) is mediated by transcriptional corepressors, nuclear receptor corepressor (N-CoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT). *Mol. Endocrinol.* **11**, 714-724.

Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A. H., Osuga, J., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T., Ishibashi, S., and Yamada, N. (1999). Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J. Biol. Chem*. **274**, 35832-35839.

Sirito, M., Lin, Q., Deng, J. M., Behringer, R. R., and Sawadogo, M. (1998). Overlapping roles and asymmetrical cross-regulation of the USF proteins in mice. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3758-3763.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

Stein, B., and Yang, M. X. (1995). Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. *Mol Cell Biol*. **15**, 4971-4979.

Stroup, D., Crestani, M., and Chiang, J. Y. (1997). Orphan receptors chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and retinoid X receptor (RXR) activate and bind the rat cholesterol 7alpha-hydroxylase gene (CYP7A). *J. Biol. Chem.* **272**, 9833-9839.

Subramaniam, N., Cairns, W., and Okret, S. (1998). Glucocorticoids repress transcription from a negative glucocorticoid response element recognized by two homeodomain-containing proteins, Pbx and Oct-1. *J. Biol. Chem*. **273**, 23567-23574.

Sugiyama, T., Wang, J.-C., Scott, D. K., and Granner, D. K. (2000). Transcription activation by the orphan nuclear receptor, chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI). *J. Biol. Chem.* **275**, 3446-3454.

Swift, G. H., Liu, Y., Rose, S. D., Bischof, L. J., Steelman, S., Buchberg, A. M., Wright, C. V., and MacDonald, R. J. (1998). An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through formation of a trimeric complex with PBX1b and MRG1 (MEIS2). *Mol Cell Biol.* **18**, 5109-20.

Tae, H. J., Zhang, S., and Kim, K. H. (1995). cAMP activation of CAAT enhancer-binding protein-beta gene expression and promoter I of acetyl-CoA carboxylase. *J. Biol. Chem*. **270**, 1487-1494.

Thurmond, D.C. and Goodridge, A.G. (1998). Characterization of thyroid hormone response elements in the gene for chicken malic enzyme. Factors that influence triiodothyronine responsiveness. *J. Biol. Chem*. **273**, 1613-1622.

Tong, G. X., Tanen, M. R.; and Bagchi, M. K. (1995). Ligand modulates the interaction of thyroid hormone receptor beta with the basal transcription machinery. *J. Biol. Chem*. **270**, 10601-11061.

Tran, P., Zhang, X.-K., Salbert, G., Hermann, T., Lehmann, J. M., and Pfahl, M. (1992). COUP orphan receptors are negative regulators of retinoic acid response pathways. *Mol. Cell. Biol.* **12**, 4666-4676.

Usala, S. J., Menke, J. B., Watson, T. L., Wondisford, F. E., Weintraub, B. D., Bérard, J., Bradley, W. E. C., Ono, S., Mueller, O. T., Bercu, B. B. (1991). A homozygous deletion in the c-*erb*Aα thyroid hormone receptor gene in a patient with generalized thyroid hormone resistance: isolation and charcterization of the mutant receptor. *Mol. Endocrinol.* **5**, 327-335.

Vallet, V. S., Henrion, A. A., Bucchini, D., Casado, M., Raymondjean, M., Kahn, A., Vaulont, S. (1997) Glucose-dependent liver gene expression in upstream stimulatory factor 2 -/- mice. *J. Biol. Chem*. **272**, 21944-21949.

Vinson, C. R., Hai, T., and Boyd, S. M. (1993). Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev*. **7**, 1047-1058.

Wang, L.-H., Tsai, S. Y., O'Malley, B. W., and Tsai, M.-J. (1989). COUP transcription factor is a member of steroid receptor superfamily. *Nature* **340**, 163-166.

Wang, L.-H., Ing, N. H., Tsai, S. Y., O'Malley, B. W., and Tsai, M.-J. (1991). The COUP-TF composes a family of functional related transcription factors. *Gene Express.* **1**, 207-216.

Wang N., Finegold, M. H., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995). Impaired energy homeostasis in C/EBP alpha knockout mice. *Science* **269**, 1108-1112.

Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Groul, D. J., Evans, R. M. (1986). The c-*erb*A gene encodes a thyroid hormone receptor for thyroid hormone. *Nature* **324**, 635-640.

Wijnholds, J., Muller, E., and Ab, G. (1991). Estrogen facilitates the binding of ubiquitous and liver-enriched nuclear proteins to the apoVLDL II promoter *in vivo*. *Nucleic Acids Res*. **16**, 369.

Williams, G. R., Harney, J. W., Forman, B. M., Samuels, H. H., and Brent, G. A. (1991). Oligomeric binding of T3 receptor is required for maximal T3 response. *J. Biol. Chem.* **266**, 19636-19644.

Xu, L., Glass, C.K., and Rosenfeld, M.G. (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev*. **9**, 140-147.

Yano, S., Fukunaga, K., Takiguchi, M., Ushio, Y., Mori, M., and Miyamoto, E. (1996). Regulation of CCAAT/enhancer binding protein family members by stimulation of glutamate receptors in cultured rat cortical astrocytes. J. *Biol. Chem*. **271**, 23520-23527.

Yeagley, D., Moll, J., Vinson, C. A., and Quinn, P. G. (2000). Characterization of elements mediating regulation of phosphoenolpyruvate carboxykinase gene transcription by protein kinase A and insulin. Identification of a distinct complex formed in cells that mediate insulin inhibition. *J. Biol. Chem*. **275**, 17814-17820.

Yen, P.M., and Chin, W.W. (1994). New advances in understanding the molecular mechanisms of thyroid hormone action. *Trends Endocrinol*. *Metab*. **5**, 65-72.

Yu, V.C., Delsert, C., Andersen, B., Holloway, J.M., Devary, O.V., Näär, A.M., Kim, S.Y., Boutin, J.-M., Glass, C.K., and Rosenfeld, M.G. (1991). RXR□: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67**, 1251-1266.

Zenke, M., Muñoz, A., Sap, J., Vennström, B., and Beug, H. (1990). v-*erb*A oncogene activation entails the loss of hormone-dependent regulator activity of c-*erb*A. *Cell* **61**, 1035-1049.

Curriculum Vitae

Yutong Wang

Department of Biochemistry
School of Medicine
West Virginia University
P.O. Box 9142
Morgantown, WV 26506-9142

EDUCATION

Aug. 1996 to Jan. 2001	Ph.D. candidate in Department of Biochemistry, West Virginia University, Morgantown, West Virginia. Title of dissertation: "The role of accessory proteins in controlling the thyroid hormone regulation of transcription of the malic enzyme gene."
Sept. 1991 to May 1996	B.S., Department of Cell Biology and Genetics, College of Life Sciences, Peking University, China.

EMPLOYMENT

Aug. 1996 to Jan. 2001	Graduate research assistant, laboratory of Dr. Brad Hillgartner, Department of Biochemistry, West Virginia University. Research area: Regulation of transcription of lipogenic enzymes.

LABORATORY EXPERIENCE

Areas of expertise/strong familiarity:

- Genomic Library Screening
- Transient Transfection
- Thin Layer Chromatography
- DNase I Hypersensitivity Analysis
- Site-Directed Mutagenesis
- Southern Blot Analysis
- DNA Sequencing
- GST Pull-Down Assay
- Gel Mobility Shift DNA-binding Assay
- Subcloning
- PCR
- UV-VIS Spectroscopy
- Gel Electrophoresis
- DNA Isolation
- Cell Culture and Sterile Techniques
- Centrifugation

Areas of familiarity (via occasional hands-on performance):

- Enzyme Kinetics
- Gas Chromatography
- Membrane-Bound Protein Purification
- HPLC
- DNA Fingerprinting Analysis
- Microscopy
- Electron Microscopy

PRESENTATIONS

Formal Seminars:

"Regulation of transcription of the malic enzyme gene. Characterization of accessory proteins that modulate thyroid hormone responsiveness." Dissertation Proposal (1998, WVU)

"Rho GTPase and actin cytoskeleton" Seminar outside research field (1998, WVU)

"Molecular chaperone function of calreticulin" Seminar outside research field (1997, WVU)

Informal Seminars:

Biochemistry Research Forum

Van Liere Research Competition in School of Medicine, West Virginia University

Molecular and Cellular Biology Journal Club

TEACHING

Instructor for problem-solving and discussion sessions in the Human Function course (CCMD330) for first-year medical students in Fall, 1998.

Laboratory assistant for Biochemistry 139, General Biochemistry Laboratory for undergraduate students in Fall, 1997.

Tutor for medical technology students studying in the area of biochemistry.
Supervised laboratory rotations of graduate students in Dr. Hillgartner's laboratory.

AWARDS

Prattana Life Sciences Scholarship Award, 1994 and 1995 (Beijing, China).

A member of the Peking University Chess Team that won the team championship in National University and College Chess Competition in China, 1994.

PUBLICATIONS

Wang, Y., Yin, L., and Hillgartner, F. B. The homeodomain proteins Pbx and Meis1 Are accessory factors that enhance thyroid hormone regulation of the malic enzyme gene in hepatocytes. In preparation.

Wang, Y., Zhang, Y., and Hillgartner, F. B. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) enhances thyroid hormone responsiveness of the malic enzyme gene in hepatocytes. In preparation.